UNIVERSITY OF ALBERTA

BRAIN PROTEIN KINASE ACTIVITY FOLLOWING CHRONIC EXPOSURE TO ANTIDEPRESSANT DRUGS

by

Neviana G. Dimova ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of Master of Science

Division of Neuroscience

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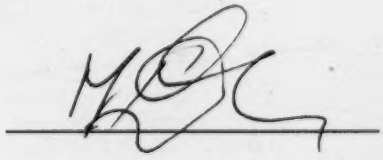

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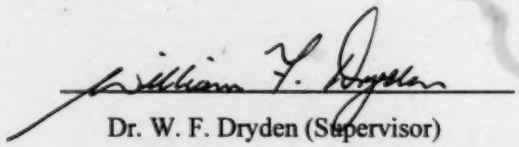

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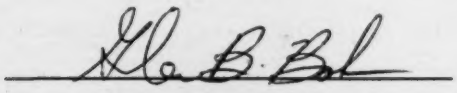

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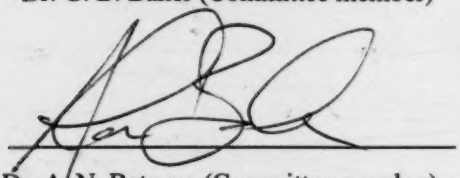

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DEDICATION

This thesis is dedicated to my parents, Elena Paskaleva and Georgi Dimov, and to my brother, Ivan for the love, support and encouragement they have provided unconditionally throughout my life.

ABSTRACT

The effects of chronic administration of different antidepressant drugs on the activities of protein kinase C (PKC) and cyclic AMP dependent protein kinase (PKA) in rat brain regions were examined. Tranylcypromine (TCP), imipramine (IMI) or fluoxetine (FLU) were infused for 14 days, and enzyme activities of cortex and hippocampus determined. Brain IMI and FLU levels, and MAO inhibition in the TCP treated brains, were determined. TCP and IMI both shifted PKC activity from soluble to membrane cortex fractions, while FLU increased activity of the soluble fraction alone. In hippocampus, FLU shifted activity from soluble to particulate fractions. PKA activity in the soluble fraction of cortex was decreased by TCP and FLU but increased by IMI. All drugs increased soluble PKA activity in hippocampus. PKA activities in particulate fractions were unaffected. MAO inhibition with TCP was >80%, and levels of IMI and FLU (and active metabolites) were about 800ng/gm and 17 μg/gm respectively.

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LIST OF ABBREVIATIONS

AKAPs	A-kinase anchoring proteins
Ach	acetylcholine
AC	adenylate cyclase
A_{595}	protein absorbance reading at wavelength 595 nm
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CaM	calmodulin
CaM kinase	Ca^{2+}/calmodulin-dependent kinase
Ci	curie
COMT	catechol O-methyl transferase
cpm	counts per minute
CRE	cAMP-response element
CREB	cAMP-response element binding protein
C-terminal	carboxyl terminal
DAG	diacylglycerol
DA	dopamine
DbcAMP	dibutyryl cAMP
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)N,N,N',N'-tetraacetic acid
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate

GTP	guanosine triphosphate
G-protein	guanine nucleotide binding protein
GTP	guanosine triphosphate
5-HT	5-hydroxytryptamine, serotonin
HCl	hydrochloric acid
HPA	hypothalamic-pituitary-adrenal axis
IP_3	inositol triphosphate
K^+	potassium ion
K_2CO_3	potassium carbonate
KCl	potassium chloride
kDa	kilo dalton
LTD	long-term depression
LTP	long-term potentiation
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
µM	micromolar
mM	millimolar
$MgCl_2$	magnesium chloride
MW	molecular weight
NaCl	sodium chloride
nM	nanomolar
NA	noradrenaline
PFBC	pentafluorobenzoyl chloride
PI	phosphatidylinositol
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PKI	protein kinase inhibitor
PMSF	phenylmethylsulphonyl fluoride
rpm	rotations per minute

SSRI	selective serotonin reuptake inhibitor
TCA	trichloracetic acid
TCAs	tricyclic antidepressants
TPA	phorbol-myristate acetate response element
Tris	Tris (hydroxymethyl) aminomethane
v/v	volume per volume
w/v	weight per volume
°C	degrees Celsius

1. INTRODUCTION

The onset of action of antidepressant medication is commonly believed to require at least two weeks, based on clinical observations. However, this time interval is not consistent with the biochemical effects of a single dose of those drugs, which usually occur within a few hours. The observed delay in clinical response to antidepressants suggests that some long-term adaptive alteration may occur in response to the immediate action of the drugs, and the adaptive changes are responsible for the improvement in mood experienced by patients.

1.1. Antidepressant drugs in the treatment of depression

1.1.1. Emerging theory and its employment in drug therapy

Basic and clinical studies in biological psychiatry have provided evidence that a relative deficiency in brain noradrenaline (NA) and/or 5-hydroxytryptamine (5-HT, serotonin) transmission (Coppen, 1967; Sulser, 1993) may play a key role in the etiology of depression. This hypothesis received initial support from the development of tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) and the fact that they increased monoamine levels in the synaptic cleft. Cerebral dopamine (DA) neurotransmission, although less often implicated in the etiology of depression, has been reported to be generally decreased in mood disorders and increased in mania (Swerdlow *et al.*, 1987; Diehl *et al.*, 1992). The role of DA in depression is supported by the fact that there are relatively selective DA reuptake inhibitors, such as bupropion, that also have some direct agonist action on DA receptors and have shown good antidepressant effects in some cases of treatment-resistant depression (Scully, 1996).

All therapies used currently in the treatment of depression have in common the ability to intensify monoaminergic availability at central synapses, although they may do so by acting through different mechanisms, e.g. inhibition of monoamine reuptake, inhibition of monoamine catabolism and increasing monoamine release.

1.1.2. The discovery of antidepressants

The group of agents known today as antidepressants emerged in late 1950s with the nearly simultaneous discovery of MAOIs and TCAs. At first research was focused on the action of these drugs at the synapse, on inhibition of MAO activity and inhibition of monoamine reuptake, respectively, but the subsequent development of the so-called novel antidepressants (e.g. iprindole, mianserin, viloxazine), which are not particularly strong inhibitors of NA or 5-HT, do not inhibit MAO and do not have the classical tricyclic structure (Sulser, 1982; 1986), raised doubts about the potential target sites for the action of antidepressants. Research then concentrated on changes in neuronal signal transduction at the receptor level produced by the antidepressants (Vetulani, 1984; Kendall *et al.*, 1985; Baker and Greenshaw, 1989), but here also controversial results were obtained, particularly with the selective serotonin reuptake inhibitor (SSRI) antidepressants (Bourin and Baker, 1996). A subsequent step in the signal transduction cascade, the regulation of the state of phosphorylation of specific substrates by a variety of protein kinases, is a general mechanism by which many hormones, neurotransmitters and other extracellular signals produce their physiologic responses in specific target cells (Nestler and Greengard, 1983; 1984). The studies of antidepressant action on protein phosphorylation-dephosphorylation in the last decade have provided interesting findings about the long-term effects of antidepressants on protein kinase activity.

1.1.3. Proposed levels of modulation

As a consequence of the research mentioned above, abnormal NA and/or 5-HT function has been hypothesized to be a result of disturbances at various pre- and postsynaptic levels, including modification of the sensitivity of receptors (Racagni and Brunello, 1984; Blier *et al.*, 1994), the function of the receptor coupled G-proteins (Menkes *et al.*, 1983), the action of protein kinases controlling several intracellular processes (Nestler *et al.*, 1989; Racagni *et al.*, 1992), and finally of the regulation of gene expression at the

nuclear level (Nestler *et al.*, 1993).

In an effort to explain the etiology of affective illness, an imbalance in the levels of activities of the second messengers has been proposed as responsible for the onset of depression. Watchel (1989) suggested that depression may be related to reduced function of the cyclic AMP-dependent protein kinase A (PKA) cascade, connected with the β-adrenoceptor, with concomitant domination of the calcium/calmodulin-dependent kinase II (CaM kinase II) and protein kinase C (PKC) pathways which are connected with the muscarinic M_1 cholinergic receptor.

1.2. Biochemical pathology of depression

1.2.1. Diagnostic criteria for major depression

Major depression is relatively common psychiatric disorder, with a 1-year prevalence rate estimated at 10.3% for major depressive episodes recently and a lifetime prevalence of 17.1% in the general population in the USA (Kessler *et al.*, 1994). The lifetime risk for depression ranges from 10 to 25% for women and from 5 to 12% for men. The lifetime prevalence is estimated to be approximately 6% in the general population (American Psychiatric Association, 1994). In The Diagnostic and Statistical Manual (vol. IV) of the American Psychiatric Association (DSM-IV), depression is defined as consisting of five or more of the following signs or symptoms persisting for more than a two week period: depressed mood, reduced interest in obtaining pleasure, weight loss or gain, anorexia, sleep disorder, psychomotor agitation or retardation, fatigue, feelings of inappropriate guilt, diminished ability to concentrate and recurrent thoughts of death. The above symptoms may produce significant impairment of a patient's life.

1.2.2. Hypothalamic-pituitary-adrenal axis involvement

A number of depressed patients have repeatedly demonstrated biochemical abnormalities, comprising sustained activation of the hypothalamic-pituitary-adrenal (HPA) axis (Holsboer and Barden, 1996; Nemeroff, 1998), the system that controls the body's response to stress, and increased concentrations of corticotropin releasing hormone (CRH) in the cerebrospinal fluid (Nemeroff *et al.*, 1984; Holsboer *et al.*, 1986; Nemeroff, 1996). There is physiological evidence for abnormalities of hormone release and for circadian fluctuations of pituitary gland hormones in depressed patients (Souetre *et al.*, 1986; Deuschle *et al.*, 1997). A hypersecretion of cortisol is one of the clinical markers of depression (Gibbons, 1964; Meltzer *et al.*, 1987; Barden *et al.*, 1995). A proposed association of central glucocorticoid receptors with altered neurotransmitter function arose from the observation that these receptors are present on the nuclei of catecholamine- and serotonin-containing cells in the brain (Tuomisto and Mannisto, 1985). Although the most important regulatory mechanism for the HPA system is an autoregulatory feedback by corticosteroids, its activity can also be enhanced by NA, 5-HT and acetylcholine, whereas GABA inhibits it (Tuomisto and Manisto, 1985).

Glucocorticoid receptors have been shown to function as DNA binding proteins that can regulate gene transcription of the β-adrenoreceptor and 5-HT receptors (Hadcock and Malbon, 1988; Kuroda *et al.*, 1994). On the other hand, a diverse range of neurotransmitters can modulate the HPA system. It has been found that long-term treatment with various TCA drugs (desipramine, clomipramine, imipramine) causes an increase in glucocorticoid receptor immunoreactivity (Kitayama *et al.*, 1988; Shimoda *et al.*, 1988) in noradrenergic and serotoninergic cell bodies in rat brain and suppression of the HPA system. A suggestion has been made that antidepressants normalize raised cortisol secretion (Silverstone and Turner, 1995; Barden *et al.*, 1995; Holsboer and Barden, 1996) and indirectly, noradrenergic and serotoninergic neurotransmission, by up-regulating the desensitized central glucocorticoid receptors (Leonard, 1997).

1.2.3. Diverse theories

Diverse theories for the etiology of mood disorders have been put forward, although none of them explains in full the observed neurotransmitter fluctuations in the disease state. Among them, functional increases or decreases in several neurotransmitter systems in addition to NA and 5-HT, e.g. acetylcholine (Janowsky *et al.*, 1972), γ-aminobutyric acid (GABA) (Petty *et al.*, 1992), and DA (Diehl and Gerson, 1992) have been pointed out.

One of the observations provided by geneticists is that depression and manic-depression frequently run in families. It has been hypothesized that certain genetic traits indirectly diminish monoamine levels in CNS synapses or increase the reactivity of the HPA axis to stress (Nemeroff, 1998). Adoption studies have yielded consistent evidence for genetic susceptibility to depression. Linkage studies (Newman and Holden, 1993; Berrettini, 1995) indicate that loci on chromosome 18, 21 and X could contribute to genetic predisposition to depression. In addition there is evidence for a contribution to the risk of depression from environmental factors, such as electromagnetic fields and certain chemicals (Verkasalo *et al.*, 1997; Bonhomme-Faivre *et al.*, 1998).

1.2.4. Monoaminergic transmission

The chemical pathology of depression has been extensively investigated, with a principal focus on changes in biogenic amine and amino acid precursor concentrations and neurotransmitter receptor function and density (Pandey *et al.*, 1991; Hrdina and Vu. 1993; Goodnough and Baker, 1994; Pandey *et al.*, 1997; Duman *et al.*, 1997; Nemeroff, 1998).

A large experimental data base suggests alterations in serotonergic and noradrenergic transmission in depression (Owens and Nemeroff, 1994). Serotonin is believed to play an important role in psychiatric disorders, which is to be anticipated from its involvement in the physiological processes of sleep, mood, feeding and possible sexual behaviour and learning, all of which are deranged in depression (Leonard, 1997). Another piece of evidence is the

high therapeutic effectiveness of TCAs and, more recently, SSRIs, which block presynaptic 5-HT reuptake transporters. Depletion of 5-HT affects neurons in diverse brain regions as well as indirectly dampening NA signalling (Sanders-Bush and Canton, 1995). Seven major families of 5-HT receptors with at least 15 subpopulations have now been identified (Glennon and Dukat, 1995); these are linked to two major signal transduction pathways: activation of guanine nucleotide binding proteins (G proteins), followed either by a multistep enzyme mediated pathway or a direct regulation of ion channels (Sanders-Bush and Canton, 1995).

1.2.4.1. Involvement of 5-HT receptors

Receptors of the 5-HT_1 group are found in high density in the mammalian hippocampus, the septum, the amygdala and the cortical limbic area. 5-HT_{1A}, 1_B and 1_D receptors are mainly negatively coupled to an adenylyl cyclase cascade through the G_i family of G-proteins. The best-characterized representative of that group, the 5-HT_{1A} receptor, in addition directly enhances voltage-sensitive K^+ channel activity and inactivates Ca^{2+} channels, therefore depressing neuronal firing rate. Receptors of the 5-HT_2 group, 5-HT_{2A}, 2_B and 2_C (formerly 5-HT_{1C}) on the other hand are widely distributed throughout the brain, with highest density in the neocortex, and appear to play a role in appetite control, thermoregulation and sleep. Various neuroleptic agents and antidepressants bind with relatively high affinity at 5-HT_{2A} receptors. 5-HT_2 receptors are directly coupled to a phosphoinositol second messenger system activation through a $G\alpha_q$ subunit and $\beta\gamma$ subunits derived from G_i and G_o (Lesch *et al.*, 1991; Duman *et al.*, 1989; Bourin and Baker, 1996), and by mobilizing diacylglycerol and/or free calcium ions, they can activate either PKC or a CaM kinase (Sanders-Bush and Canton, 1995).

Each cascade in its turn regulates the activity of a great number of cellular proteins by phosphorylation-dephoshorylation. Radioligand binding techniques have shown evidence of functional interactions between 5-HT_1 and 5-HT_2 receptors in depression (Fuxe *et al.*, 1983; Deakin, 1991). Some 5-HT_2 antagonists additionally bind at DA receptors with lower

affinity (Glennon and Dukat, 1995; Leonard, 1997). Deakin *et al.* (1991) suggested that 5-HT_1 and 5-HT_2 receptors are affected in opposite directions after long term administration of TCAs, MAOIs, SSRIs and some of the novel antidepressants and by chronic electroconvulsive treatment, with down-regulation of 5-HT_2 receptors and up-regulation of 5-HT_1 receptors (Newman and Lerer, 1988; Baker and Greenshaw, 1989; Nalepa, 1994). However there are some inconsistencies in the literature with regard to SSRIs and electroconvulsive treatment (Hrdina and Vu, 1993; Goodnough and Baker, 1994; Hyttel, 1994; Todd *et al.*, 1995; Bourin and Baker, 1996).

1.2.4.2. G-protein transducers

G proteins play a central role in coupling membrane receptors to various intracellular effector systems. They have been extensively studied in the recent years (Milligan, 1993; Wilcox and Gonzales, 1995), particularly with regard to psychiatric disorders (Manji, 1992; Hudson *et al.*, 1993; Milligan, 1993). The molecular mechanism by which neurotransmitters produce their intracellular effects is interaction of G-proteins with either adenylate cyclase (AC) (responsible for production of cAMP) or phospholipase C (PLC) (producing phospoinositol second messengers) enzymes. PLC is implicated in coupling with G_q, G_i and G_o, depending on the cell type. AC is stimulated by $G\alpha_s$ as well as by an increase of intracellular Ca^{2+}, and inhibited by $G\alpha_i$ and/or G_o. Free $\beta\gamma$ subunits, while in abundance, have been found also to contribute to AC inhibition at least for the some forms of the enzyme (AC type I and calmodulin-sensitive enzyme) (Duman and Nestler, 1995; Antoni *et al.*, 1998).

1.2.4.3. Adrenergic receptors

There is experimental evidence of an association of deficits of the catecholamines NA and DA with depression and locomotor retardation. NA is the main catecholamine in

postganglionic sympathetic nerves and in certain areas of the central nervous system. Recently adrenaline has been shown to be a transmitter in the hypothalamus (Leonard, 1997). Adrenergic projections innervate thalamus and dorsal hypothalamus, hippocampus and subcortical limbic regions.

All noradrenergic receptors produce their effects through activation of G-proteins. They comprise of α- and β-subfamilies (Newman and Lerer, 1989; Duman and Nestler, 1995). All β-adrenoreceptors are found in abundance in brain; β_1-receptors are mainly found in heart, β_2- and α_1- in smooth and skeletal muscle and β_3- in adipose tissue (Duman and Nestler, 1995). β_1-, β_2- and β_3-receptors all produce their physiological effects via interaction with G_s and the subsequent stimulation of the PKA cascade. It was originally thought that α_2-adrenoreceptors are mainly presynaptic in peripheral postganglionic sympathetic nerve fibers, mediating the presynaptic inhibition of NA release and coupled with G_i to inhibition of AC. There is recent evidence that they occur also postsynaptically in the CNS (Leonard, 1997), in close proximity to β-receptors (Pilc and Enna, 1985; Nalepa and Vetulani, 1991; Nalepa, 1994) and could produce α- mediated β-receptor potentiation of neuronal responses. With the development of molecular cloning techniques, more subclasses of α_2- receptors have been discovered: α_{2A}-, α_{2B}-, α_{2C}- (Duman and Nestler, 1995). α_2-Adrenergic receptors are linked to AC through G_i/G_o protein, and it has been hypothesized that they mediate the inhibition of the PKA cascade. α_1- Adrenergic receptors are coupled through G_i/G_o or G_q to the phosphoinositol second messenger cascade and hence to the PKC cascade. All β- receptors are widely distributed in brain and are linked through G_s protein to the PKA cascade (Nicholls *et al.*, 1992; Sanders-Bush and Canton, 1995).

1.2.4.4. Dopaminergic receptors

Dopaminergic neurons were first discovered in caudate nucleus and putamen and have been implicated in the control of motor activity. Later it was discovered that DA receptors are widely dispersed in most brain areas.

The central dopaminergic system can be divided into three major subdivisions: nigrostriatal, tuberoinfundibular and mesocortical/mesolimbic systems. The nigrostriatal system projects from substantia nigra to the caudate and putamen and is involved in the movement control. The tuberoinfundibular system projects from the arcuate and periventricular nuclei into the intermediate lobe of pituitary and the median eminence and appears to be responsible for regulation of the secretion of such hormones as prolactin.

The mesocortical and mesolimbic systems consist of long fibres which originate from the ventral tegmental area and terminate in the limbic cortex (septum, nucleus accumbeus, nucleus of the stria terminalis, amygdaloid complex and piriform cortex), the lateral septal nuclei, the medial frontal, anterior cingulate and entorhinal cortex. It is thought that limbic structures mediate complex emotional responses as fear anxiety and pleasure. Some investigators believe that the cingulate gyrus plays a dual role in emotional reactions and in memory processing. All these projections are functionally important in psychotic disorders and in eliciting the effects of neuroleptic drugs (Leonard, 1997).

Dopamine receptors consist of two families, the D_1-like (D_1 and D_5 receptors) and the D_2-like (D_2, D_3 and D_4 receptors). They have been studied generally in connection with neuroleptic effects. The D_1/D_5 type of receptors are coupled with G_s and result in AC stimulation and subsequent activation of PKA and concomitant release of parathyroid hormone (Kebanian and Calne, 1979). Their activation has been found to hyperpolarize neurons, although the specific ion channels involved have not yet been identified (Duman and Nestler, 1995). The first D_2 receptors were located in anterior and intermediate lobes of the pituitary from which DA inhibits the release of prolactin and α-melanocyte-stimulating hormone (Stoof and Kebanian, 1984). The D_2-D_4 receptors are coupled to G_i/G_o proteins and inhibit AC and PKA cascade. It has been found that D_1 type of receptors are approximately 15 times more sensitive to the action of DA than D_2. D_1 may thus provide the background tone which may either synergize or oppose the actions of D_2 receptors. Seeman and coworkers (1989; 1990) have suggested that there is a link between D_1 and D_2 receptors that is mediated by G proteins and which may be missing in some patients with schizophrenia.

1.2.5. Effects of antidepressant drugs on G-proteins

Chronic administration of antidepressants does not change the total amount of G proteins, although it has been found to reduce the expression of G_s and G_i protein α-subunits (Duman *et al.*, 1989; Lesch *et al.*, 1991; Lesch and Manji, 1992) and to cause an overall increase of G_o proteins, particularly in cerebral cortex. There is an increased amount of active $G\alpha_s$/AC complexes, suggesting that chronic antidepressant treatment alters certain membrane components such that a greater proportion of $G\alpha_s$ is activated (Ozawa and Rasenick, 1989; Chen and Rasenick, 1995; Dwivedi and Pandey, 1997). It has been found that fluoxetine decreases $G\alpha_s$ expression in midbrain, while $G\alpha_q$ and $G\alpha_{12}$ expression was increased in neostriatum and frontal cortex (Lesch *et al.*, 1992). In addition TCAs, fluoxetine and zimelidine have been found to facilitate AC activation (Menkes *et al.*, 1983; Ozava and Rasenick, 1989; Newman *et al.*, 1990; 1992), although Yamaoka *et al.* (1987) found a dose-dependent inhibition of AC *in vitro* by a large number of TCAs.

1.3. Second messenger-regulated protein kinases

Phosphorylation is a posttranslational covalent modification used by prokaryotic and eukaryotic cells to control the properties of a wide variety of proteins, including enzymes, receptors, ion channels and regulatory or structural proteins (Nestler and Greengard, 1984; Walaas and Greengard, 1991; Nestler and Greengard, 1994). Protein kinases are phosphotransferases which catalyze the transfer of the γ-phosphoryl group of ATP to an amino acid residue in the presence of Mg^{2+}. Two general classes of these enzymes exist, serine/threonine kinases, which transfer phosphate to serine or threonine residues (PKC, PKA, PKG and Ca^{2+} /calmodulin dependent kinase) and those transferring phosphate to tyrosine residues (tyrosine kinases).

1.3.1. Protein kinase A

The mechanism of activation of a number of membrane receptors involves regulation of the formation of cAMP and subsequent activation of PKA. With the exception of some ion channels regulated directly by cAMP, all known effects of cAMP in eukaryotic cells are conferred by PKA. The cAMP pathway involves hormone receptors that transduce their signal across the cell membrane via G proteins that interact with membrane-bound AC to either increase or reduce the production of cAMP. This mechanism has been implicated in a number of cellular processes such as metabolism, gene regulation, cell growth and division, cell differentiation, sperm motility, ion channel conductivity and neurotransmitter release (McKnight, 1991; Scott, 1991).

1.3.1.1. Molecular structure

PKA is primarily a cytoplasmic enzyme and is present in a number of isoforms, highly enriched in mammalian CNS and with similar levels in all regions of the brain (Walaas *et al.*, 1983). It exists as a tetramer composed of two regulatory (R) subunits (molecular mass 49-55 kDa), joined by a disulfide bond, and two catalytic (C) subunits (molecular mass 40 kDa), all of which, in the absence of cAMP, bind together to produce an inactive complex. When the holoenzyme is activated by binding of two cAMP molecules to each R subunit, the catalytic subunit is released from the complex and can phosphorylate not only cytoplasmic substrates but it can also migrate into the nucleus and phosphorylate proteins important for the regulation of gene transcription (McKnight, 1991; Doskeland *et al.*, 1993; Walsh and Van Patten, 1994), such as cAMP-response element binding protein (CREB) (Hagiwara *et al.*, 1993).

From the above statements it could be concluded that the R subunit serves as a cytoplasmic anchor that prevents the catalytic subunit from entering the nucleus. The R subunit consists of an amino-terminal (dimerization) domain that is important for docking

of the enzyme to specific cytoplasmic locations, an autoinhibitory (pseudosubstrate) region and two tandem cAMP binding domains (Doskeland *et al.*, 1993; Taylor and Radzio-Andzelm, 1994). Of the two tandem cAMP-binding sites, only the second site is exposed in the inactive tetrameric PKA comlex. Binding of cAMP to this site enhances binding of cAMP to the first site in a positively cooperative fashion as a result of conformational change in the molecule (Doskeland *et al.*, 1993).

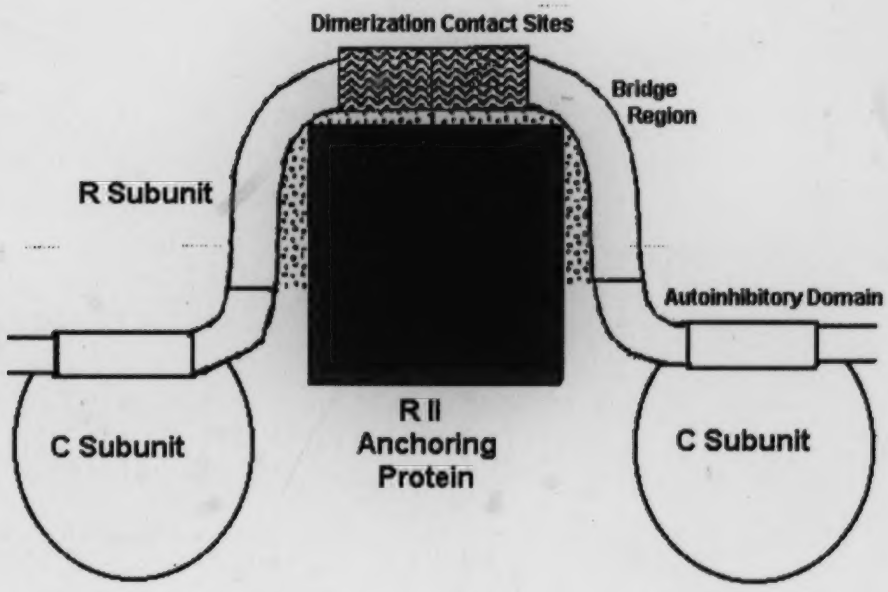

Figure 1-1. Model of the anchored type II PKA holoenzyme. Two R subunits dimerize with two C subunits, forming the inactive PKA holoenzyme. (modified from Scott *et al.*, 1990)

All of the catalytic subunits have catalytic core motifs that are common to all protein kinases and involve a Mg-ATP binding site as well as a peptide binding site. In addition, the active site contains unique structured water molecules that constitute a conserved structural element (Shaltiel *et al.*, 1997) and can play an important role in determining specificity, state of activation and cellular localization of the enzyme.

1.3.1.2. Isozymes

Two principal isozymes of PKA are present in most tissues, and these contain different R subunits: type I contains RI subunits and type II contains RII subunits. Currently four R subunits (RIα , RIβ, RIIα, RIIβ) and three catalytic subunits (C α, C β, C γ) have been identified, with at least one (Cβ) demonstrating alternate splice variants. R α and C α, subunits demonstrate widespread tissue distribution, while R β and C β isoforms are mainly localized to mammalian brain (Walter *et al.*,1978; Walaas and Greengard 1991; Tasken *et al.*, 1997). C γ has the most limited expression and has been found only in testis (Beebe *et al.*, 1990).

The PKA I and PKA II holoenzymes have been reported to have distinct biochemical properties. RIβ holoenzymes are 2-fold to 7-fold more sensitive to cyclic nucleotides than the RIα (Solberg *et al.*, 1994). The RIα and RIIβ genes are also subject to differential regulation by PKC (Tasken *et al.*, 1992; 1997). Several studies suggest that RIα acts as a buffer for PKA activity. For example, when C subunits are overexpressed in cultured cells, RIα increases to regulate PKA activity and when RII is overexpressed RIα decreases as the RII holoenzyme is formed preferentially (Brandon *et al.*, 1997). In addition, RII subunits are generally found in the particulate fraction of neural homogenates, attributable to their high affinity for A-kinase anchoring proteins (AKAPs). In contrast, RI subunits tend to be found in the cytosolic fraction of cells, although this is not absolute. Autophosphorylation of RII, which is stimulated by cAMP (Walaas and Greengard 1991), retards reassociation of the type II holoenzyme and thereby enhances its response to cAMP. Anchoring proteins

that specifically bind the RII subunits could allow for substrate selection based upon subcellular localization.

In contrast to most other tissues in which PKA is found almost exclusively in the soluble fraction, mammalian brain has high activity of the enzyme in both particulate and soluble fractions, with highest specific activities in the synaptic membrane and cytosol fractions. Within brain, total PKA activity is highest in basal ganglia and cortical regions and lowest in brain stem and spinal cord (Walaas *et al.*, 1983).

The expression of different PKA subunits is subject to regulation by peptide hormones and cAMP (Landmark *et al.*, 1991) as well as by glucocorticoids and vitamin D (Berg *et al.*, 1994). In turn, PKA can regulate transcriptional activity of various genes through distinct, cAMP-inducible promoter-responsive sites (Ziff, 1990; Borrelli *et al.*, 1992).

1.3.1.3. Regulation of gene expression and memory potentiation by PKA

Many genes have CRE, a DNA binding motif, in their promoters, which confers stimulation by cAMP on gene transcription. CRE is activated on binding of a soluble protein-nuclear factor cAMP-responsive element binding protein (CREB). When activated, the catalytic subunit of PKA can travel to the nucleus and phosphorylate CREB at serine/threonine residues. Currently more than 10 CREB-factors activating several genes have been cloned. There are both activators and repressors of cAMP-responsive transcription. Some alternatively spliced CRE modulator (CREM) isoforms act as antagonists of cAMP-induced transcription in testes. The different factors can heterodimerize with each other or with Fos and Jun proteins in certain combinations (Monaco *et al.*, 1997). On dephosphorylation by protein phosphatase, CREB is deactivated. Recently it has been reported that RIIβ also on its own can bind to CRE and exhibit transcriptional activity (Srivastava *et al.*, 1998) as an alternative pathway for direct regulation of transcription.

Another important function of the PKA system investigated recently is its involvement in memory and learning and other forms of neural plasticity in response to long-term treatment with pharmacological agents. Mutant mice lacking one or the other of R or C subunits have shown defects in long-term potentiation (LTP) and long-term depression (LTD) in visual cortex and nociception (Brandon *et al.*, 1997).

1.3.1.4. Protein kinase inhibitors

Many tissues contain thermostable protein kinase inhibitor protein (PKI) with short sequences similar to a pseudosubstrate site of the enzyme, which can bind and inactivate the free C subunit (Walsh *et al.*, 1971; Ashby and Walsh, 1972; Walaas and Greengard 1991) and has been shown to promote the movement of the catalytic subunit of PKA from the nucleus to the cytoplasm, probably regulating the nuclear actions of PKA on gene expression (Fantozzi *et al.*, 1994).

1.3.1.5. PKA effects and substrates in central nervous system

The following points are of significance:

1. PKA is localized to the cytoskeleton and activated by interaction with MAP 2 (Akiyama *et al.*, 1986; Scott *et al.*, 1990), which is further phosphorylated by PKA, but at a different amino acid sequence than by PKC. It is proposed that it may also phosphorylate other specific RII-binding proteins in cells, such as lamin B and plectin (Foisner *et al.*, 1991).

   Several proteins implicated in the docking of exocytotic vesicles and neurotransmitter release have been found to be phosphorylated by PKA in vitro. A prominent substrate in brain is synapsin I (Huttner *et al.*, 1981, 1983) which binds to microtubules and microfilaments. It is thought that phosphorylation of synapsinI regulates the availability of synaptic vesicles for exocytosis by controlling the

attachment to cytoskeleton. In addition DARPP-32 while phophorylated may function as phosphatase inhibitor (Hemmings *et al.*, 1984; Nimmo and Cohen, 1987).

2. Several classes of plasma membrane receptors are found to be phophorylated by PKA. Recent studies have shown that ion channel-coupled receptors exhibiting significant similarity in their structure and membrane topology such as $GABA_A$, nicotinic acetylcholine and possibly others respond with either increased rate of rapid desensitization (Huganir *et al.*, 1986; Hopfield *et al.*, 1988) or increased response to ligand binding (Virjayaraghavan *et al.*, 1990) on phophorylation by PKA. The activity of a variety of G-protein coupled receptors (such as β-adrenergic, dopamine, muscarinic acetylcholine and serotonin receptors) is also modulated by PKA (Burgoyne, 1983; Lefkowitz *et al.*, 1990). Tyrosine kinase-coupled receptors, and particularly insulin receptor activity, might also be modulated by phosphorylation with PKA (Stadtmauer and Rosen, 1986).

3. When phosphorylated by PKA different types ion channels (Na^+, K^+, Ca^{2+}) increase both the amplitude and the duration of their corresponding currents (Rossi and Catterall, 1987, 1989; Kaczmarek, 1987, 1988; Pezolo and Bezanilla, 1990).

Although a large number of newly identified proteins have shown PKA phosphorylation sites *in vitro* (Nairn *et al.*, 1985) many of them do not satisfy the criteria for physiological PKA substrates (Krebs., 1985; Scott, 1991). Therefore the most prominent area of current studies on PKA effects is phosphorylation of transcriptional factors and its implication for gene regulation.

1.3.2. Protein kinase C

1.3.2.1. Molecular structure

The concentration of PKC, the second most abundant kinase in the nervous system, is high in brain compared to many other tissues, suggesting that this enzyme plays an important role in the regulation of neuronal activity. The PKC enzyme family is involved

in variety of cellular processes, such as proliferation, differentiation, secretion and tumorigenesis (Blumberg, 1991). At present it is thought to consist of 12 different members, indicated by the Greek symbols α, βI, βII, γ, δ, ε, ξ, η, θ, ι, λ, and μ (Dekker and Parker, 1994; Nishizuka, 1988; 1989; 1995). The enzymes are highly enriched in both cytoplasmic and membrane preparations from vertebrate and invertebrate brain (Kuo *et al.*, 1980; DeRiemer *et al.*, 1985) and are highly conserved among different species (Dekker and Parker, 1994). They are composed of a single subunit protein of 77-83 kDa and are divided into Ca^{2+}-dependent and -independent subgroups of phospholipid/diacylglycerol (DAG)-stimulated serine/threonine kinases.

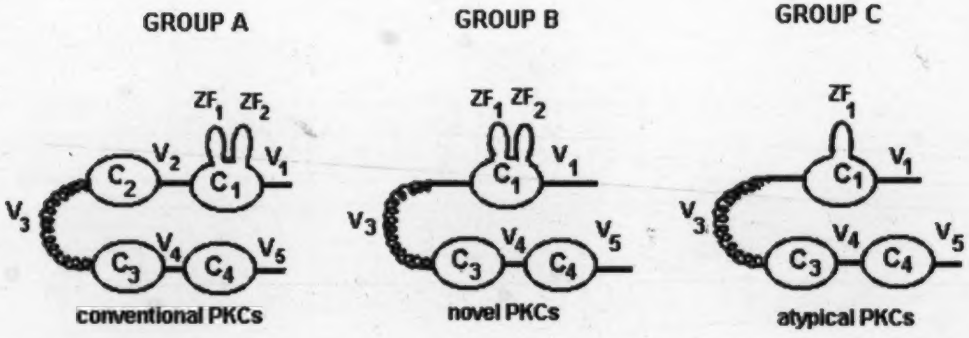

Figure 1-2. Structure of PKC isozymes. The different isoforms have been classified into three groups according to structural and functional differences (modified from Kazanietz and Blumberg, 1996)

1.3.2.2. Physiological activators of PKC

Many neurotransmitters and hormones activate a receptor-linked phospholipase C (PLC) which catalyzes the hydrolysis of membrane phosphatidylinositol (PI) (Walaas and

Greengard, 1991) and is mainly involved in the formation of DAG within the plasma membrane. This pathway is believed to be the major physiological pathway for the activation of PKC, although other pathways are present as well, including breakdown of phosphatidylcholine (Billah and Anthes, 1990) or of inositol-containing glycolipids (Saltiel *et al.*, 1986), fatty acids and arachidonate derivatives (Huang, 1989; Khan *et al.*, 1995). DAG greatly increases the enzyme's affinity for Ca^{2+} in the micromolar range. However in physiological conditions this concentration is nanomolar (Takai *et al.*,1979a,b). Phospholipids, together with DAG, further increase the affinity of PKC for Ca^{2+}. It is hypothesized that Ca^{2+} ions provide the contact for the enzyme on binding to the phosholipids (Bazzi and Nelsestuen, 1993).

1.3.2.3. Mechanism of activation

The binding of the phospholipid to the protein is highly sequential and each step is irreversible upon completion (Bazzi and Nelsestuen, 1991). There are two intermediate steps: first is the formation of a ternary enzyme+Ca^{2+}+phospholipid complex and second is binding of DAG or phorbol ester and further promotion of activation.

In the presence of phospholipids, DAG and Ca^{2+} there is a conformational change in the regulatory domain of PKC. That is followed by activation and association of the enzyme with the plasma and organelle membranes, which is observed as translocation of enzyme activity from the cytoplasm to the plasma membrane (Kraft and Anderson, 1984; Kaczmarek, 1987).

It has been hypothesized that sustained activation may produce a constitutively active kinase or create a transmembrane ion channel (Bazzi and Nelsestuen, 1993). On the other site, prolonged activation results in the eventual proteolytic degradation or down-regulation of the enzyme (Huang & Huang 1992; Azzi *et al.*, 1992). A number of structurally diverse tumor promoters (such as phorbol esters, aplysiatoxins, teleocidines, bryostatins, ingenols) also activate PKC (Nakamura *et al.*, 1989; Blumberg, 1991) in a fashion similar to that of DAG, implicating the enzyme in tumorigenesis.

1.3.2.4. Isoforms

Members of the PKC family are divided into conventional, novel and atypical (Huang and Huang, 1992; Nishizuka, 1995; Harrington and Ware, 1995), exhibiting some differences in their biochemical characteristics and substrate specificities. All of the members of the family require PS for activation. The conventional (α, βI, βII and γ) isoenzymes of PKC, which were discovered first, have conserved structural motifs with a high degree of sequence homology within the group as well as in different species. They comprise four conserved sections of polypeptide chain (C_1-C_4) and five variable ones (V_1-V_5). The C_1 polypeptide section of all PKCs exept ξ-PKC contains a tandem repeat of a cysteine-rich sequence, with a zinc finger within it, which was originally thought to function as a DNA binding motif and is found in many transcription factors. In the PKC family it is responsible for binding of DAG and phorbol esters (Ono *et al.*, 1989; Nakanishi and Exton, 1992). In the beginning of the C_1 domain there is a pseudosubstrate sequence, an amino acid sequence that closely resembles PKC substrate recognition sites and when the enzyme is inactive is tightly bound to the catalytic site. The C_2 domain binds Ca^{2+} with a so-called EF hand motif (Parker *et al.*, 1986). The amino terminal half of each polypeptide containing C_1 and C_2, as well as V_1 and V_2 and part of V_3 is the regulatory domain that interacts with Ca^{2+}, Zn^{2+}, phospholipids and DAG or phorbol esters. The carboxy-terminal half is the catalytic domain and contains the sections C_3, C_4 (or the ATP and substrate binding sites) and V_4 region (Hug and Sarre, 1993; Nishizuka, 1995; Harrington and Ware, 1995).

Recently discovered novel PKC isozymes (δ, ε, η, θ, and the closely related μ) lack the C_2 section and do not require Ca^{2+} for activation (Dekker and Parker, 1994; Hug and Sarre, 1993; Nishizuka, 1995). PKC activity can be regulated by autophosphorylation, which has been reported for all enzymes known (Huang and Huang,1992; Hug and Sarre, 1993; Nishizuka, 1995; Bazzi and Nelsestuen, 1992) or phosphorylation (both PKC δ and ε can be phosphorylated by other protein kinases). Both the regulatory and the catalytic domains were found to be autophosphorylated, which alters the affinity of the enzyme for phorbol esters and increases its sensitivity to Ca^{2+} and its rate of histone H1 phosphorylation.

The atypical ξ-and λ(ι)-PKC also lack the C_2 region and contain only one cysteine-rich sequence. The ξ-isoform is slightly different than the rest of the family in the pseudosubstrate site and exhibits a constituitive protein kinase activity that is dependent on phosphatidylserine (PS) and cis-unsaturated fatty acids (oleic, linoleic, linolenic, arachidonic) for catalytic activation in vitro, but does not respond to DAG and phorbol esters (Huang and Huang,1992; Nishizuka, 1995; Harrington and Ware, 1995). The λ- and the ι-isoforms appear to be variants of a single entity and have a high degree of identity with the ξ-isoform.

1.3.2.5. Regulation by other proteins

The activity of PKC also is subject to regulation by other endogenous proteins. In vitro, PKC can be degraded by trypsin or calpain into two fragments, containing a catalytic and a regulatory domain. The protease sensitive sites are located within the hinge region between these two domains. PKC could be cleaved further into two fragments by an endogenous Ca^{2+}-dependent protease in rat brain, and the larger fragment generated that way is catalytically active in the absence of phospholipids, DAG or Ca^{2+}, resulting in irreversible activation of the enzyme (Kaczmarek 1987).

Recently it has been found that PKC α, δ and βI isozymes may be directly activated or inhibited by phosphorylation on a tyrosine residue (Denning *et al.*, 1993, Li *et al.*, 1994) Cross-talk between the PKC family and the MAP (mitogen-activated) kinases pathway has been discovered and can activate at different points Ras and Raf proteins, products of the early oncogenes RAS and RAF (Tanaka and Nishizuka, 1994).

1.3.2.6. Tissue and cellular distribution

All PKC isoenzymes are widely distributed in tissues and a single cell may produce a variety of isoforms, depending on the stage of development. The α, β, δ, ε, λ, μ and ξ isoenzymes are distributed in brain, heart, muscle, lung and skin (Hug and Sarre, 1993).

PKC γ has been found mainly in nervous system and adrenal tissues, PKC θ is expressed chiefly in skeletal muscle, hematopoetic cells, platelets and endothelium, and η is strongly expressed in skin and lung and only slightly in brain and spleen (Chang *et al.*, 1993; Baier *et al.*, 1993). PKC α and δ are distributed widely in all tissues and cell types and could control nuclear functions at specific points of the cell cycle.

With regard to cellular and subcellular localization, PKC α and β seem to be located mainly in the cytosol and on activation translocate to either the nucleus or the plasma membrane (Hug and Sarre, 1993) whereas ξ is found mainly in cell nucleus. Within the brain, highest PKC activity has been found in cortical regions, including the hippocampus, and in the cerebellum and lowest activity has been found in the brain stem and spinal cord (Walaas and Greengard, 1991). It has been shown that different isoenzymes may become expressed at a different stages of mammalian development; therefore, well-coordinated regulation of PKC activation appears to be important for normal cell functions (Harrington *et al.*, 1995; Nishizuka, 1995; Kazanietz *et al.*, 1996).

1.3.2.7. Regulation of gene transcription by PKC

Phorbol esters, the typical PKC activators, have been shown to induce changes in gene transcription. A specific sequence in DNA called TPA (phorbol-myristate acetate)-responsive element has been discovered which could bind to activator proteins of the c-jun and c-fos families of the early oncogenes Fos and Jun and induce the transcription of specific genes (Kazanietz and Blumberg, 1996). Unlike the direct interaction between CREB and PKA, the interaction between c-jun and PKC is indirect and results in dephosphorylation of c-jun and c-fos, probably by a nuclear phosphatase that has been activated by phosphorylation by PKC. In contrast to the CRE system phosphorylation here plays a negative role.

The distribution, translocation and down-regulation of PKC may be visualized by the analysis of the so-called particulate (i.e. membrane) and soluble (i.e. cytosolic) fractions of the cells and further activity measurement or immunoblotting. In this regard, translocation

to the cellular membranes has been regarded as the activation of PKC (Kazanietz and Blumberg, 1996; Deckker and Parker, 1994).

1.3.2.8. PKC effects and substrates in the central nervous system

PKC exhibits diverse effects on separate levels of modulation of: cell shape, axonal growth, cellular contacts, mitotic response and signaling through modulation of ion channel activity and neurotransmitter release. All these effects are achieved through phosphorylation of a great number of protein substrates, ion channels and receptor proteins.

PKC phosphorylates a diverse range of cellular proteins, a great number of which bind to a significant fraction of total cellular calmodulin (CaM) and release it on phosphorylation (on an increase of free Ca^{2+}). CaM binds strongly to neuromodulin (Andreasen *et al.*, 1983; Alexander *et al.*, 1987), neurogranin (Baudier *et al.*, 1991), and the MARCKS protein (Graff *et al.*, 1989) probably by interacting with their basic clusters (Houbre *et al.*, 1991). When the serine residues in those basic clusters are phosphorylated by PKC (Graff *et al.*, 1989; Baudier *et al.*, 1991) CaM binds very weakly (Alexander *et al.*, 1987; Graff *et al.*, 1989) and is readily released from those proteins. Since the concentration of neuromodulin and MARCKS protein in brain cells is sufficient to complex a significant fraction of the total CaM, several groups (Alexander *et al.*, 1987; Liu and Storm, 1990; Houbre *et al.*, 1991) have hypothesized that those proteins may act as "molecular switches", phosphorylated by PKC, and controlling the free concentration of CaM at specific locations in cells. This hypothesis was further extended by Skene (1989) and Liu and Storm (1990) who suggested that neuromodulin binds and concentrates CaM, especially on neural growth cone membranes, and that after phosphorylation by PKC, the released CaM may interact with cytoskeletal proteins. Therefore the polymerization, crosslinking and membrane attachment of cytoskeleton polymers might be affected, causing "softening" of the membrane and increased filopodia formation and extension in neuronal growth cones. It has been demonstrated that neuromodulin is present and concentrated in axons of hippocampal neurons in culture (Goslin *et al.*, 1988), but not in growing dendrites. In addition it synthesis

synthesis and accumulation decline an order of magnitude as animals mature (Jacobson *et al.*, 1986). Neuromodulin may give the axons the ability to elongate and sprout over long distances in response to environmental clues.

Another cytoskeletal protein, MAP 2, is also phosphorylated by PKC as well as by PKA (on different residues than by PKC). On association with microtubules and microfilaments it induces tubulin polymerization and neuronal microtubule formation. When phosphorylated by PKC, that ability is greatly decreased (Akiyama *et al.*, 1986; Hoshi *et al.*, 1988).

One of the initially discovered substrates for PKC, MARCKS is also widely distributed in neuronal pseudopodia and filopodia and colocalized with other cytoskeletal members, vinculin and talin (Graff *et al.*, 1989; Rosen *et al.*, 1990; Thelen *et al.*, 1991). Its phosphorylation by PKC is accompanied by cell spreading and loss of filopodia.

Another protein which plays a role in determining the form and shape of neurons and is also a substrate for PKC is adducin (Gardner and Bennett, 1987; Bennett and Lambert, 1991). It binds tightly to spectrin-actin complexes and promotes assembly of additional spectrin molecules onto actin filaments (Bennett *et al.*, 1988). This ability is abolished by macromolar concentrations of CaM and Ca^{2+}.

Further, but not of least importance, PKC plays a role in mitosis, cell growth and differentiation. It may also phosphorylate, in a dose dependent manner, several nuclear envelope polypeptides, the predominant target identified as lamin B and plectin (phosphorylated also by PKA) (Fields *et al.*, 1988, 1989, 1990; Foisner *et al.*, 1991). Since phosphorylation of lamin B is known to be involved in nuclear lamina depolymerization at the time of mitosis, this could explain the effects of PKC on cell growth and proliferation. Taken together, these findings suggest that the former proteins play a role in interlinking cytoskeletal and nuclear elements or in alterations of cellular metabolism occurring with mitosis.

Nuclear enzymes such as DNA polymerase β and DNA topoisomerase I are also phosphorylated by PKC. When phosphorylated, DNA polymerase β is inactivated (Tokui *et al.*, 1991), which may be an important process in the modification of DNA metabolism in

the nucleus through signal transduction processes. On the other hand when phosphorylated DNA topoisomerase I is activated (Pommier *et al.*, 1990) and relax supercoiled DNA processively, which may play role in the duplication of DNA before mitosis begins.

Cellular effects of PKC extend also to the regulation of neurotransmitter release and ion channel activity (Kaczmarek, 1986; 1987). Activation of PKC in a cells of Aplysia bag cell neurons results in striking enhancement of calcium action potentials evoked by depolarizing stimuli (DeRiemer *et al.*, 1985; Nickols *et al.*, 1987) and increase of Ca^{2+} concentration in separate cellular compartments, which is believed to trigger neurotransmitter release. On the other side phosphorylation of anchoring proteins also may induce exocytosis from synaptic vesicles of already synthesized neurotransmitters. Taken together that suggests the role of PKC in enhancement through separate but probably converging pathways of neurotransmitter release.

Finally PKC produces its effects on regulation of gene expression through indirect action of TPA and activator proteins, product of c-Jun and c-Fos genes and through intermediate steps of phosphorylation or dephosphorylation.

1.4. Antidepressant drugs

1.4.1. Development of the concept for antidepressants

The high affinity, energy-dependent mechanism of reuptake of the biogenic amines NA, DA and 5-HT is of primary importance for terminating the physiological action of these transmitters at their receptor sites. Logically, drugs that inhibit the reuptake mechanism would prolong the physiological action of the transmitters. Cocaine and amphetamines are such drugs that inhibit the reuptake of the catecholamines. Nevertheless, despite the action of these drugs in prolonging the intersynaptic activity of the neurotransmitters there is no evidence that they have antidepressant properties. Most of the conventional and novel antidepressants are potent inhibitors of NA and/or 5-HT reuptake yet there is no evidence to prove conclusively that the potency and specificity of action in inhibiting the amine reuptake

is related to their therapeutic effect.

Currently there are several major groups of antidepressants: TCAs and tetracyclic antidepressants, MAOIs, SSRIs, triazolopyridines and the relatively novel bupropion and venlafaxine.

1.4.2. Tricyclic antidepressants

The TCAs and the MAOIs are considered classical antidepressant drugs. The TCAs are potent inhibitors of NA and/or 5-HT reuptake in nerve terminals both in vitro and in vivo, thus resulting in increased bioavailability of the neurotransmitter amines at central receptors. Although TCAs produce their biochemical action (inhibition of amine reuptake) soon after drug administration begins, significant antidepressant effects are usually not observed until after at least a two-week period (which correlates with the period of down-regulation of β-adrenergic and 5-HT_2 receptors observed with several antidepressants) (Nalepa and Vetulani, 1991; Baker *et al.*, 1992; Goodnough and Baker, 1994).

All TCAs have a three-ring nucleus in their molecular structures. Imipramine, amitriptyline, clomipramine, trimipramine and doxepin are tertiary amines and they have two methyl groups on the nitrogen atom of the side chain. Desipramine, nortriptyline and protriptyline are secondary amines having only one methyl group in that position. The tertiary amines are metabolized into their corresponding secondary amines (e.g. desipramine, nortriptyline) in the body (Silverstone and Turner, 1995; Leonard, 1997), with the tertiary amines being more potent 5-HT, and the secondary amines more potent NA, reuptake inhibitors. After administration of a tertiary amine, high concentrations of its corresponding secondary amine are achieved, therefore no TCA could be considered to be a selective 5-HT reuptake inhibitor. Oral absorption of most TCAs is incomplete and there is a significant metabolism from the first pass-effect, whereby the drug passes via the portal system directly to the liver (Silverstone and Turner, 1995). Protein binding is usually over 75%,and the TCAs are highly lipid soluble.

Side effects of TCAs have been attributed to blockade of muscarinic acetylcholine,

histamine and α-adrenergic receptors. Anticholinergic effects (dry mouth, blurred vision, tachycardia), sedation, autonomic effects and cardiac effects are common adverse effects of TCAs, as is weight gain with prolonged treatment (Kaplan and Sadock, 1993).

1.4.3. Monoamine oxidase inhibitors

1.4.3.1. MAO enzymes

Two principal enzymes are involved with biogenic amine degradation and thus may have a role in antidepressant effects. These are MAO and catechol O-methyltransferase (COMT). Whereas there are several specific MAOIs used for treatment of depression, inhibitors of COMT have found little application. MAO-A preferentially oxidizes NA and 5-HT and MAO-B preferentially oxidizes β-phenylethylamine in low concentrations. At high concentrations of substrates both enzymes oxidize all the substrates nonpreferentially. The two forms of MAO are widely distributed in both nervous and non-nervous tissue. Non-selective inhibition of MAO by drugs such as phenelzine leads to an increase in the bioavailability of several biogenic amines in the synaptic cleft, including trace amines such as tryptamine, β-phenylethylamine and adrenaline (Leonard, 1997). It has been suggested that the positive effect of MAOIs is associated with the enhanced availability of these amines in brain (Silverstone and Turner, 1995; Leonard, 1997).

MAO-A is widely distributed in all tissues whereas MAO-B predominates in human brain and platelets (Leonard, 1997). The older MAOIs such as phenelzine and tranylcypromine are nonselective (inhibit both MAO-A and MAO-B) and irreversible, while some newer MAOIs such as moclobemide, brofaromine and toloxatone are reversible MAO-A inhibitors.

1.4.3.2. Adverse effects of MAOIs

The most frequent adverse effects of MAOIs are orthostatic hypotension, weight gain, sexual dysfunction and insomnia. A major problem with all irreversible MAO-A inhibitors is the so-called "cheese effect", caused by ingesting tyramine-rich foods while taking the MAOIs (Baker *et al.*, 1992), which inhibit gut MAO-A. This inhibition of tyramine metabolism is followed by an increase of tyramine (a potent sympathomimetic amine) in blood and subsequent NA release from nerve terminals, causing symptoms which may range from headache to a major hypertensive reaction (and possibly death). This adverse effect appears to be much less of a problem with reversible MAO-A inhibitors. The cheese effect is not a problem with (-)-deprenyl at doses at which it inhibits only MAO-B, but (-)-deprenyl is not a particularly effective antidepressant. Its use has been largely restricted to Parkinson's disease (Leonard, 1997). When administered with SSRIs (fluoxetine, fluvoxamine, paroxetine, sertraline), MAOIs may produce a "serotonin syndrome" consisting of autonomic instability, hyperthermia, rigidity, myoclonus, confusion, delirium and coma (Kaplan and Sadock, 1993).

1.4.4. Selective serotonin reuptake inhibitors

As a result of findings suggesting that the serotonergic system is disrupted in depression, a number of SSRIs were introduced. They have no significant effect on muscarinic, histaminergic or adrenergic receptors and therefore exhibit fewer side effects than TCAs and MAOIs. Trazodone and nefazodone, 5-HT uptake inhibitors, also have partial serotonin-receptor agonist properties and venlafaxine inhibits uptake of 5-HT as well uptake of NA and to a lesser extent of dopamine (Ellinrod and Perry, 1994; Andrews *et al.*, 1996).

1.4.5. Experimental data from receptor studies

Using studies of receptor binding, it has been demonstrated that chronic administration of TCAs, MAOIs, SSRIs and some of the novel antidepressants and chronic electroconvulsive treatment induce a reduction of the number of 5-HT_2 and β-adrenoreceptors in rat forebrain (Fuxe *et al.*, 1983; Newman and Lerer, 1988; Baker and Greenshaw, 1989; Nalepa and Vetulani, 1991; Nalepa, 1994), and up-regulation of 5-HT_{1A} receptors in rat hippocampus (Fuxe *et al.*, 1983; Blier *et al.*, 1990), although there are some inconsistencies in the literature, particularly with regard to SSRIs and electroconvulsive treatment (Hrdina and Vu, 1993; Goodnough and Baker, 1994; Bourin and Baker, 1996). Studies have shown that there are several clinically effective antidepressants, particularly the SSRIs fuoxetine, citalopram or paroxetine, which do not induce down-regulation of β-adrenoreceptors, except at very high doses (Hyttel, 1994; Wong *et al.*, 1995).

1.4.6. Experimental results of protein kinase studies

Relatively few studies have examined the effects of long-term administration of antidepressants on PKA in rat brain. With long-term administration of imipramine, tranylcypromine and electroconvulsive shock, a decrease in PKA activity has been observed in the soluble fraction, but increased enzyme activity was found in the particulate fraction in rat frontal cortex (Nestler *et al.*, 1989). However, changes in the opposite directions were reported by Tadokoro *et al.* (1998). Racagni *et al.* (1992) demonstrated that desipramine increases the amount of the regulatory subunit of PKA in the soluble fraction of rat cortex. Perez *et al.* (1989) found an increased cAMP-dependent endogenous phosphorylation of microtubules in rat cerebral cortex on treatment with desipramine, and Mori *et al.* (1998) found a similar effect with SSRIs. Inhibition by a variety of antidepressants of cAMP response element-binding protein/cAMP response element-directed gene transcription have been observed by Schwaninger *et al.* (1995). Patients with untreated depression have significantly lower cAMP-dependent protein kinase activity in skin fibroblasts than do

normal subjects (Shelton *et al.*, 1996).

Reduced PKC activity has been found both in soluble and particulate cellular fractions (Kendall and Nahorski, 1985; Mann *et al.*, 1995; Morishita and Watanabe, 1997; Szmigielski and Gorska, 1997) in rat cortex and hippocampus upon treatment with desipramine and fluoxetine, although with some inconsistencies (Tadokoro *et al.*, 1998). Increased PKC activity in the cytosolic fraction of platelets of depressed patients (Pandey *et al.*, 1998) and decreased activity of PKC in the membrane and cytosolic fractions of brains of suicide victims (Pandey *et al.*, 1997) have been observed. Hrdina, on the other hand, using Western Blotting and monoclonal antibodies to detect enzyme molecules, found no significant difference in the presumed amounts of enzyme in brain regions from depressed suicides compared to matched controls (Hrdina *et al.*, 1998).

2. SPECIFIC OBJECTIVE

My objective was to study the long-term effects of exposure to different antidepressant drugs on protein kinases in the central nervous system. The protein kinases are implicated in the transduction mechanisms of many neurotransmitter receptors and there is some evidence for their dynamic changes on long-term antidepressant treatment. This evidence is scant, contradictory, selective and poorly related to clinically relevant drug concentrations. The following investigation is an effort to provide more consistent data by combining several classes of antidepressant drugs in a single study and examining the activity of PKA and PKC following chronic exposure to these drugs. In addition the levels of the drugs (or the effect on MAO activity, in the case of tranylcypromine) were monitored to ensure that the drugs had been delivered to the brain by the osmotic minipumps.

3. MATERIALS AND METHODS

3.1. Materials

Where appropriate, chemicals were at least of analytical quality. Sources are identified in the following list.

Chemicals	Supplier
Bio-Rad protein dye reagent	Biorad Laboratories, Mississauga
Whatman cation exchange paper P 81	VWR Canlab, Toronto
TCA, K_2CO_3, NaCl	Fisher, Ottawa
PKI, sucrose, histone IIs, pepstatin	"
Leupeptin, phorbol myristacetate.	"
Dithiothreitol, aprotinin, PMSF, phosphatidyl	Sigma, St. Louis
serine, EGTA, benzamidine, $SnSO_4$,	"
$MgSO_4$, Tris, β-mercaptoethanol, $CaCl_2$,	"
Mg acetate, adenosine triphoshate,	"
Dibutyrylcyclic AMP, Cytoscint®.	"
Toluene, ethyl acetate	BDH, Toronto
Acetic anhydride	Caledon Labs, Georgetown
Pentafluorobenzoyl chloride	Aldrich, Milwaukee
[γ-^{32}P]-ATP	Amersham, Toronto

Drugs	Supplier
Methophane	Janssen Pharmaceuticals, North York
Desipramine HCl, maprotiline HCl,	Sigma, St. Louis
Imipramine HCl.	"
Tranylcypromine HCl, β-phenylethylamine HCl	"
Fluoxetine HCl, fluvoxamine maleate.	Eli Lilly, Indianapolis
2-Phenyl[1-^{14}C]ethylamine hydrochloride	NEN Chemicals
(50.8 mCi/mmol)	"
5-[2-^{14}C] hydroxytryptamine binoxalate (54.7 mCi/mmol).	"

Supplies	Supplier
Osmotic minipumps	Alza Corporation, Palo Alto

All glassware used in drug extraction procedures was sonicated in a diluted Contrad® 70 (Canlab) solution for total of 60 min and was then vigorously rinsed with distilled water and dried in a convection oven before use.

3.2. Methods

3.2.1. In vivo drug treatments of animals:

Three groups of 250-300g male Sprague-Dawley rats (8 per group) were housed in pairs in an environmentally controlled room using a 12 h light-12 h dark cycle. The rats were implanted in the dorsal thoracic area with Alzet® 2 ml subcutaneous osmotic minipumps, loaded to administer imipramine (10 mg/kg/day), tranylcypromine (1 mg/kg/day) or vehicle for 14 days with a rate of delivery of 5 µl/h. Two additional groups (8 per group) were implanted two months later with fluoxetine (10 mg/kg/day) or vehicle.

All surgical instruments and wound clips were immersed for a couple of minutes in 5% Savon® disinfectant. Each rat was anesthetized with about 2 ml of Meflonate® (by inhalation). With a scalpel blade, a 2 cm long incision was made through the skin and the fascia; with the blunt end of the scissors, a pocket was made between the fascia and muscle into which the pump was inserted. The wound was clipped and topical anesthetic applied. The rat was then placed for several minutes under an incandescent lamp for heat, followed by placement in normal animal housing.

Alzet®osmotic minipumps operate because of an osmotic pressure difference between a compartment within the pump and the tissue environment in which the pump is implanted. The high osmolality of the middle compartment of the pump causes the water to flux into the pump through a semipermeable membrane, which forms the outer surface of the pump. As

the water enters the middle compartment, it compresses the flexible middle reservoir, displacing the test solution from the pump at a controlled predetermined rate. The rate of delivery by a pump is controlled by the water permeability of the pump's outer membrane. Thus, the delivery profile of the pump is independent of the drug formulation dispensed. Drugs of various molecular configurations and molecular weight can be dispensed continuously at controlled rates with a range between 0.25 to 10 µl/h.

Each day the implanted pumps were rotated and the rats checked for signs of necrosis or inflammation. The rats were killed by decapitation on the 15th day of treatment for IMI and TCP, and on the 16th day in the case of FLU, their brains dissected out and placed on ice. The cortex and the hippocampus were used for protein kinase assays. The remaining tissue was immediately frozen in isopentane cooled on dry ice or dry ice alone and was used later for analysis of drug levels.

3.2.2. Protein kinase assays

3.2.2.1. Protein kinase C assay: (Based on Mann *et al.*, 1995)

The frontal cortex was homogenized (10 mg wet weight/ml) for 30 s, using an ULTRA TURREX T25 (speed 20, 000g) in 11.17 ml of ice-cold buffer [0.32 M sucrose, 5 mM benzamidine, 2 mM dithiothreitol (DTT), 3 mM EGTA, 0.5 mM $ZnSO_4$, 0.5 mM $MgSO_4$, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 0.05 mg ml^{-1} pepstatin, 0.1 mg ml^{-1} aprotinin, 0.05 mg ml^{-1} leupeptin]. To prevent enzyme activation and protein degradation all procedures were carried out at 4°C and the protease inhibitors PMSF, pepstatin, aprotinin and leupeptin were freshly added to the buffer before each experiment.

Homogenates were then centrifuged at 10,000 g and 4° C for 8 min to disrupt nuclei and cytoskeleton, then divided into supernatant and pellet and the latter discarded. The supernatant was then centrifuged second time at 100,000 g at 4° C for 1 h. The resulting pellet, resuspended in the original volume of buffer, and the supernatant constituted the particulate and soluble fractions of the tissue, respectively. From each sample tissue was

estimated the enzyme activity in the presence of EGTA (controls), lipid only, Ca^{2+} only, and both lipid and Ca^{2+}, in triplicates, for both membrane and cytosolic fractions. Assay tubes contained (final volume of 0.12 ml): 12 µl of the incubation buffer (20 mM Tris HCl, 5 mM mercaptoethanol, 10 mM $MgCl_2$, 0.67 mg/ml histone, pH 7.5), 12 µl of 1 mM $CaCl_2$ and 12µl lipid (10 mg/ml phosphatidylserine and 3.3 µM phorbol myristacetate). In zero Ca tubes, 12µl 1mM EGTA was substituted for the $CaCl_2$, and in zero lipid tubes, lipid was simply omitted.

The reaction was initiated by the addition of 12µl radiolebelled Mg-ATP in buffer (100µCi ml^{-1} [^{32}P]-ATP, 150µM ATP, 45mM $(CH_3COO)_2Mg.4H_20$ in 50 mM Tris-HCl , pH 7.5), to each tube, except blank controls. Radiolabelled ATP solution was made up fresh for each experiment, and used over a succession of 3-4 days.

All assay tubes (in triplicate), were incubated at 37° C for 30 min. An aliquot from each tube (40 µl) was blotted on to individual 1cm Whatman (P81) ion exchange paper squares. The reaction was then terminated by placing the paper squares into a 10% trichloroacetic acid bath, stirring for 10 min once and then washing three times in 5% trichloroacetic acid, for 15 min each. The papers were further washed with running tap water for 30 min and finally, prior to air drying, immersed in 95% ethanol to denature of the remaining proteins. The retained radioactivity was determined with a Beckman LC 6500 scintillation counter (using Cytoscint® counting fluid as a medium for immersing the paper squares).

3.2.2.2. Protein kinase A assay: (Based on Nestler and Tallman, 1988)

Brains were removed rapidly from decapitated rats, dissected on ice and cooled immediately in the ice-cold buffer (the cortex into 9.5 ml and the hippocampus in 1.5 ml) used in the PKC assay. The brains were homogenized and centrifuged at 10,000 g and 4° C for 8 min to disrupt nuclei and cytoskeleton, then divided into supernatant and pellet and the latter discarded. The resulting supernatant was then centrifuged at 100,000 g and 4° C for 1 h. The supernatants were designated the soluble fractions and the pellets were

resuspended in the original volume of homogenization buffer and designated the particulate fractions. cAMP-dependent protein kinase activity was assayed in triplicates in both the soluble and particulate fractions by using a standard histone phosphorylation-filter paper assay (Witt and Roskovski, 1975a,b) as described above, with dibutyrylcyclic AMP (DBcAMP) 5 μM, as the enzyme stimulator or PKI (Sigma P 5015) 50 μg/ml, as enzyme inhibitor. In these assays, PKA activity was calculated as the level of histone phosphorylation observed in the presence of PKI, a specific inhibitor of this protein kinase, subtracted from the level of cAMP stimulated enzyme. This was based on the premise that many other kinases are also stimulated by the addition of cAMP, but only PKA specifically is inhibited by PKI.

3.2.2.3. Calculation

Enzyme activity was calculated as follows. Background counts were initially subtracted from the count for each sample. Due to the short half life of ^{32}P, the specific activity of the ATP for each day was determined from a 10μl sample of solution counted separately, and the activity of 1 pM ATP established. This was then applied to the counts from each tube to determine the amount of phosphate retained on the ion exchange papers as phosphorylated histone. This was then extrapolated to determine the amount of phosphate transferred per ml of the original brain extract in the reaction tube. From the measurement of protein content of the brain extracts, soluble and particulate, the amount of phosphate transferred per mg protein in the original extract could be determined. The calculations thereafter diverged, depending on the enzyme. For PKC, the values obtained from the zero Ca^{2+} and the zero lipid tubes were subtracted from the mean of the three triplicates of test solutions. The resulting values indicated the degree of Ca^{2+}- or lipid-dependent activity present in the test samples. There was little difference in the two values, and the values quoted in the results are the Ca^{2+}-dependent activity. For PKA, the activity determined in the PKI-containing tubes was subtracted from the activity in the test samples. The difference represented activity that was susceptible to specific inhibition, and is quoted in the results

as PKA activity.

3.2.3. Protein content measurement (based on the method of Bradford, 1976 and modified by Read and Northcote, 1981).

For determination of the protein content in each sample, a Bio-Rad Protein Microassay (adapted from the method of Bradford) was used. A range of 1.25-25 µg/ml protein (utilizing 800µl of sample and 200µl of Bio-Rad reagent) was measured and spectrophotometric readings were taken at a 595nm wavelength. The principle of the assay is based on the color change of Coomassie brilliant blue G-250 dye when added to various concentrations of protein. The protonated form of the dye has a pale orange-red color, whereas the unprotonated form is blue. When the dye binds to primary basic and aromatic amino acid residues, their positive charges suppress the protonation and a blue color results. The intensity of color is measured spectrophotometrically and the results compared to a standard curve prepared using known concentrations of protein. Bovine serum albumin was used as the protein standard with concentrations ranging from 1 to 100 µg/ml. The original method of Bradford (1976) used phosphoric acid and the absorbance is measured at a single wavelength of 595 nm (A_{595}), which is the wavelength for peak absorbance of this dye in the unprotonated form.

The microassay employed here uses 200 µl of Bio-Rad reagent (dissolved initially 1/5 in distilled water) for each 800 µl of each protein sample. Color development takes place within less than 5 minutes of the addition of the dye. The readings are stable for at least 1 hour.

3.2.4. Drug analyses

3.2.4.1. Assay for imipramine and desipramine in brains of rats

Imipramine, desipramine and the internal standard (maprotiline) were determined by gas chromatography with nitrogen-phosphorus detection based on the procedure of Drebit et al. (1988). Analysis of drug levels by the chromatographic procedure was based on derivatization of desipramine and maprotiline with acetic anhydride under basic aqueous conditions followed by extraction with ethyl acetate of the drugs of interest. In this assay underivatized imipramine and acetylated desipramine can be extracted into a relatively nonpolar organic phase under conditions of basic pH. After evaporation of the solvent under nitrogen, the residue is dissolved in toluene, and portions of this solution are injected into the gas chromatograph for analysis.

Brain tissue was homogenized in 5 volumes of ice-cold distilled water. The assay was carried out in glass tubes with 1 ml of distilled water and 1 ml of homogenized brain tissue in each, using maprotiline (500 ng) as an internal standard. Sufficient 25% K_2CO_3 (w/v) was added to adjust the pH value of the solution to 11.0-11.5. This step was followed by extraction with 4 ml toluene and brief centrifugation for separation of the layers. The upper, organic layer was retained and taken to dryness under a steam of nitrogen. The samples were reconstituted in 2 ml distilled water and acetylated with acetic anhydride to derivatize desipramine and maprotiline in the presence of sodium bicarbonate in excess (small residue at the bottom of the reaction tube, resulting in a bubbling reaction mixture). When the bubbling had virtually ceased, the aqueous layer was poured into clean tubes and extracted with 5 ml ethyl acetate. The ethyl acetate (top) layer was removed to another set of tubes and taken to dryness under nitrogen. The resultant residue was reconstituted in 200 µl toluene. Aliquots of 2 µl were injected into the GC for analysis and the ratio of imipamine and acetylated desipramine to acetylated maprotiline determined. A standard curve consisting of known varying amounts of imipramine and desipramine and the same amount of maprotiline as added to the brain samples was carried through each assay run in

parallel. The ratios mentioned above for the brain samples were plotted on a standard curve and the amount of imipramine and desipramine in each sample determined.

Instrumental analysis A Hewlett Packard (HP) model gas chromatograph equipped with a nitrogen-phosphorus detector and linked to a HP 3392A integrator was used. A fused silica capillary column (25 m. X 0.32 mm. i.d.) coated with a 0.52μ-m film thickness of 5% phenylmethyl silicone (HP Co., Palo Alto, CA) was employed. The carrier gas was pure helium (Linde, Union Carbide) at a flow rate of 2 ml/min. The detector was purged with pure hydrogen (Linde, Union Carbide) at 3.5 ml/min. mixed with dry air (Linde, Union Carbide) at 80 ml/min. Injection port and detector temperatures were 250°C and 350°C, respectively. The oven temperature was programmed to increase from 105°C (maintained for 0.5 min.) at a rate of 10°C/min. to 270°C.

3.2.4.2. Assay for MAO in brains of rats treated with tranylcypromine

Determination of MAO activity was based on a modification of the procedure of Lyles and Callingham (1982). The radiochemical assay method is based on two stages - oxidation of the radiolabelled amine substrate to an aldehyde, which is subsequently oxidized to an acid with release of ammonia during the oxidation and re-oxidation of the reduced enzyme cofactor in the presence of oxygen with subsequent release of hydrogen peroxide at neutral pH. When hydrochloric acid is added the reaction is stopped, the enzyme protein is denatured and the pH value lowered so that the remaining (unmetabolized) amine becomes charged. When a solvent mixture of ethyl acetate and toluene is added to the reaction mixture, the uncharged aldehyde and acid, but not the charged amine will be extracted into the organic solvent. A portion of the solvent layer is pipetted into a scintillation vial and counted to determine the amount of radioactive aldehyde and acid present. The procedure is a direct measurement of the level of MAO activity present in the original sample of tissue homogenate. The substrates used for MAO-A and -B are radiolabelled 5-HT and β-phenylethylamine, respectively.

The assays were carried out in triplicate in borosilicate glass culture tubes. To each tube on ice was added 50 µl of brain tissue homogenate (brain tissue homogenized first in 5 volumes ice cold distilled water and than diluted to 1:160 in 0.2 M potassium phosphate buffer, pH 7.8). Subsequently, to each tube were added 50 µl radiolabelled 5-[2-^{14}C]-hydroxytryptamine binoxalate for the MAO-A assay and 2-phenyl[1-^{14}C]ethylamine hydrochloride for the MAO-B assay suitably diluted in the same potassium phosphate buffer to give final concentrations of 50 µM and 10 µM, respectively. To blank tubes were also added 10 µl of HCl (3 M). All the samples were flushed briefly with oxygen and rubber stoppers were put on quickly. The tubes were placed in a water bath at 37°C for 10 min, then removed onto ice. To each (except blanks) was added 10 µl of HCl (3 M), followed by 1 ml of ethyl acetate/toluene (1:1 v/v, water-saturated). The samples were vortexed briefly twice. That step was followed by centrifugation at 1600 rpm for 30 seconds. A portion (700 µl) of the upper organic layer was carefully pipetted into a small scintillation vial containing 4 ml of ReadySafe® scintillation fluid and the samples were counted for 3 minutes in a liquid scintillation counter Beckman LC 6500.

3.2.4.3. Assay for fluoxetine and norfluoxetine in brains of rats

Fluoxetine (FLU) and norfluoxetine (NORFLU) were determined by a gas chromatographic procedure with electron-capture detection (GC-ECD), based on the method of Rotzinger et al. (1997).

The method of analysis is based on the simultaneous formation and extraction of derivatives of FLU and NORFLU with pentafluorobenzoyl chloride (PFBC) under aqueous conditions. On extraction with toluene/PFBC in basic conditions, a chemical reaction takes place between the amine portion of FLU and NORFLU and of PFBC, hydrochloric acid is released and the pentafluorobenzoyl moiety is attached to the amine group of both drugs. The resultant highly lipophilic derivatives were extracted into toluene, a portion of that solution is analyzed by GC-ECD.

In this assay fluvoxamine (FVX) 250 ng was added as an internal standard to each

sample. Brain tissue was weighed and homogenized in five volumes of ice-cold double-distilled water. All samples were then basified (pH 11.5-12) by the addition of approximately 0.5 ml of 25% K_2CO_3 (w/v). The drugs were derivatized and extracted with 4 ml of a solution of toluene:PFBC (1 ml:0.001 ml). All tubes were then shaken vigorously for 15 min on an Ika Vibrex VXR vortex mixer and centrifugated at 1000g for 5 min in a benchtop centrifuge (Sorval GLC-2B, DuPont, USA). The organic phase was pipetted into test tubes and dried in a Savant evaporator (Speed Vac SC 110, Fisher, Ontario) for approximately 30 min The remaining residue was reconstituted in 300 µl of toluene and 1 µl of each sample was injected onto the GC.

Using known varying concentrations of FLU and NORFLU, and a fixed amount of FVX, a standard curve was run in parallel with each assay. The peak height ratios of FLU and NORFLU to FVX were compared to the values from the curve.

Instrumental Analysis: A Hewlett Packard (HP) Model 5890 gas chromatograph equipped with a ^{63}Ni electron-capture detector and linked to a HP 3392A integrator/printer was employed for the analysis of samples. A fused silica capillary column (25 m. X 0.32 mm) coated with 1.05-µm film thickness of 5% phenylmethylsilicone was used. The carrier gas was pure helium at a flow rate of 3 ml/min. The make-up gas was argon-methane (95:5) at a flow rate of 35 ml/min. The oven temperature was set at an initial temperature of 105°C for 0.5 min. The temperature was then increased by 10°/min to 225°C, followed by a 1°/min increase to 270°C. Injection port and detector temperatures were 270°C and 325°C respectively. All injections were carried out using the splitless mode of injection with a purge off time of 0.5 min.

3.2.5. Statistical analysis

Data were subjected to one-way ANOVA followed by the Newman-Keuls multiple comparison test and Bartlett's test for equal variances or Student's unpaired t-test with Welch's correction. Significant differences were considered to be established when $p<0.05$.

4. RESULTS

Rat studies of antidepressant effects are recognized as most convenient mammalian model for studying the mechanism of action of antidepressant drugs and physiological basis of depression, although it is equally widely recognized that antidepressants show few signs of their clinical activity in "normal people". Responsiveness to chronic administration of the drug of interest is taken as a requirement for an animal study of antidepressant effects (Willner, 1989). However, few animal models of depression have a time course appropriate for studying chronic antidepressant administration. The present study addresses not so much the pathophysiology of the condition, but rather the biochemical changes that are a consequence of exposure to drugs of demonstrated clinical benefit. Sprague-Dawley rats have been used here as an animal model and the experimental results are highly reproducible.

4.1. Kinase activity

The effects of antidepressants on PKA and PKC activities were determined after exposure for 14 days to delivery of drug solutions by subcutaneously implanted Alzet® osmotic minipumps with steady delivery rates of the drugs studied. The time of sacrifice was chosen to minimize residual drug interference with the assays and are based on the difference in plasma half-lives among the drugs (Nestler and Tallman, 1988; Man *et al.*, 1995; Tadokoro *et al.*, 1997). The plasma half-life of IMI is 24 h, of FLU 1-3 days and in the case of irreversible MAOIs such as tranylcypromine, at least one week is needed for synthesis of MAO-A and -B enzymes. Therefore the animals were sacrificed on the 15th day of treatment for IMI and TCP, and on the 16th day in the case of FLU. The control animals were implanted in parallel with corresponding volumes of 0.9 % saline as a vehicle. The influence of chronic antidepressant administration was examined in soluble and particulate fractions of rat cortex and hippocampus.

4.1.1. PKC activity in cortex

Two groups of experimental results, normalized to their own controls (TCP and IMI vs. controls and FLU vs. controls) were combined after meeting the requirements if Fisher's F test, and are presented in Fig. 4-1. The variance of the combined control value was obtained from each control value normalised to its own mean and incorporated together in a calculation of standard error of the mean. As shown in Fig. 4-1 the mean total activity of PKC in cerebral cortex as a percentage of control values was significantly increased with IMI and TCP treatments by ~80 %, and not significantly changed with FLU treatment. At the same time a parallel decrease of ~50 % in PKC activity of the cytosolic fraction was observed with IMI and TCP, and an increase of ~70 % with FLU. One-way ANOVA confirmed that these effects of antidepressants on PKC activity in both fractions were significant (see Table 4-1).

4.1.2. PKA activity in cortex

Two groups of normalized experimental results were combined in Fig. 4-2, (TCP and IMI vs. controls and FLU vs. controls). As shown in Fig. 4-2 an increase in the total mean activity of PKA in the cortical cytosolic fraction was observed with IMI treatment (to about ~80 %; of control value). In contrast there was a decrease with TCP and FLU. There was no change in the activity of the particulate fraction of PKA in the cortex with any of the three drugs studied. The observed shift of activity among different treatments was analyzed with one-way ANOVA and significant difference from control values occurred only in cytosolic fractions (see Table 4-1).

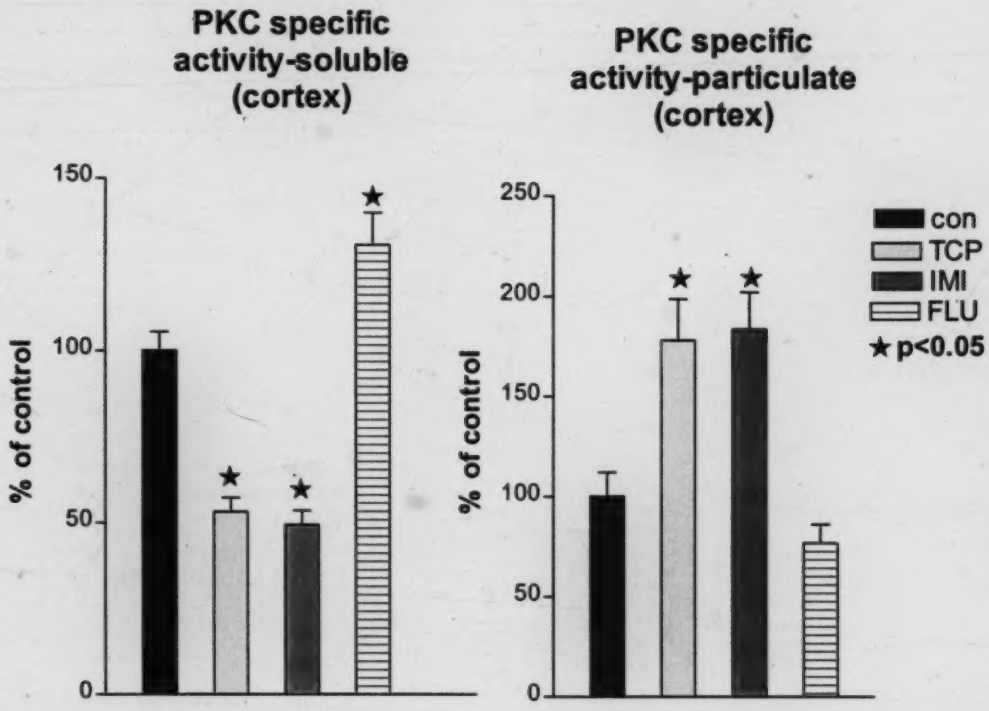

Figure 4-1. Regulation of PKC activity by different antidepressants in rat frontal cortex. Rats were treated chronically with TCP, IMI or FLU for 14 days as described in Materials and Methods. Data were normalized as a percentage of the control mean for that particular experiment, and each group is presented as mean ± SEM. Statistical significance was calculated against the appropriate experimental control group. For illustrative purposes only, the control columns represented in this figure were obtained from a combination of the values of individual control preparations normalized to the mean of its own group, and the mean and SEM calculated for the total population of control values. Data that are significantly different from their own control (ANOVA, $p<0.05$) are indicated (★). PKC activity in the particulate fraction was increased with TCP and IMI, but was unchanged with FLU. On the other hand, PKC activity in the soluble fraction was increased with FLU but decreased with TCP and IMI.

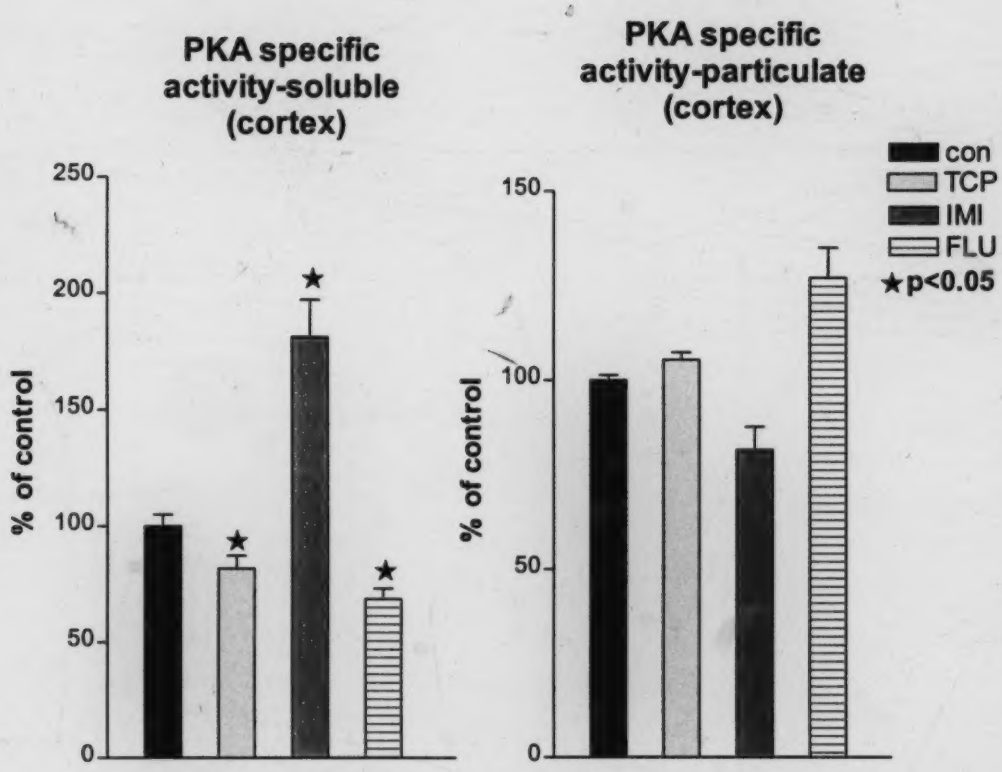

Figure 4-2. Regulation of PKA activity by different antidepressants in rat frontal cortex. Rats were treated chronically with TCP, IMI or FLU for 14 days as described in Materials and Methods. Data were normalized as a percentage of the control mean for that particular experiment, and each group is presented as mean ± SEM. Statistical significance was calculated against the appropriate experimental control group. For illustrative purposes only, the control columns represented in this figure were obtained from a combination of the values of individual control preparations normalized to the mean of its own group, and the mean and SEM calculated for the total population of control values. Data that are significantly different from their own control (ANOVA, $p<0.05$) are indicated (★). Particulate PKA activity does not show a significant change with any of the drugs investigated; PKA soluble activity, however, was increased with IMI, and decreased with both TCP and FLU.

4.1. 3. PKC activity in hippocampus

To study the effects of antidepressants further, homogenates from hippocampus were investigated for PKA and PKC total specific activities. The sparsity of tissue precluded studies on IMI and TCP, and only FLU was investigated. PKC activity in the hippocampus was decreased in the cytosolic fraction, and increased by about 50% in the particulate fraction with FLU as shown in Fig. 4-3 and Table 4-1. The observed shift of activity was analyzed with one-way ANOVA and showed statistical significance. Mann *et al.* (1995) and Morishita and Watanabe (1997) observed a decrease in PKC of both hippocampal cytosolic and particular fractions on treatment with DMI and TCP under similar experimental conditions.

4.1.4. PKA activity in hippocampus

Two groups of experimental results were combined and are presented in Fig. 4-4, (TCP and IMI vs. controls and FLU vs. controls). Mean PKA activity in the cytosolic fraction from hippocampus was increased on treatment with all three drugs studied (~150 % increase with TCP and IMI and ~ 50 % increase with FLU). In the particulate fraction there was no significant change in PKA activity with any of the drugs studied. The observed shift of activity was analyzed with one-way ANOVA and showed statistical significance.

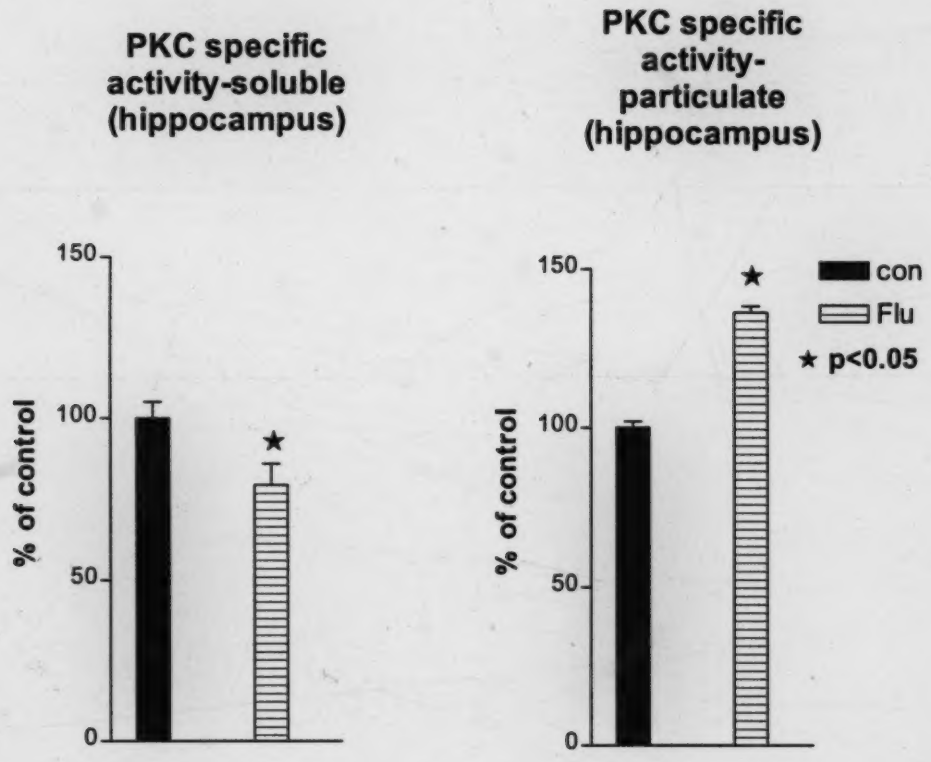

Figure 4-3. Regulation of PKC activity by FLU in rat hippocampus. Rats were treated chronically with FLU for 14 days as described in Materials and Methods. Data were normalized as a percentage of the control mean for that particular experiment, and are presented as mean ± SEM for each group. Statistical significance was calculated against the experimental control group. Data that are significantly different from their control (Student's t-test, $p<0.05$) are indicated (★). PKC activity was increased in particulate and decreased in soluble fractions of hippocampus.

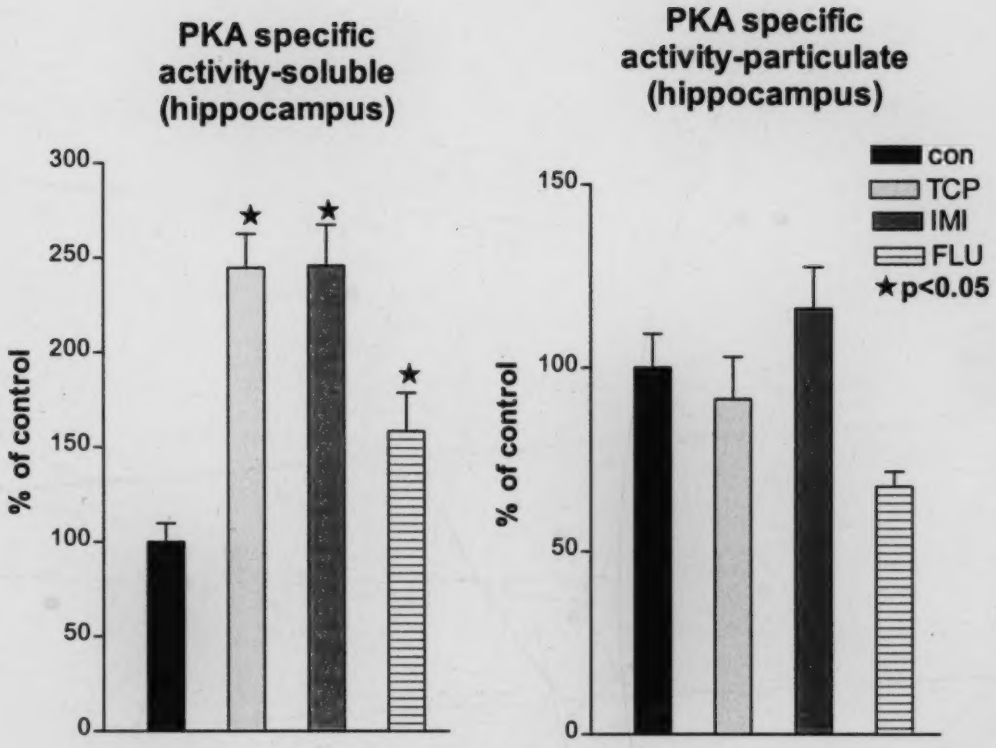

Figure 4-4. Regulation of PKA activity by different antidepressants in rat hippocampus. Rats were treated chronically with TCP, IMI or FLU for 14 days as described in Materials and Methods. Data were normalized as a percentage of the control mean for that particular experiment, and each group is presented as mean ± SEM. Statistical significance was calculated against the appropriate experimental control group. For illustrative purposes only, the control columns represented in this figure were obtained from a combination of the values of individual control preparations normalized to the mean of its own group, and the mean and SEM calculated for the total population of control values. Data that are significantly different from their own control (ANOVA, $p<0.05$) are indicated (★). PKA particulate activity does not show changes with TCP, IMI or FLU treatment. An increase in the soluble PKA activity is seen with all three drugs.

The absolute values of enzyme activities expressed in pmol phosphate transferred /mg protein over 30 minutes in the various experimental groups are presented in Table 4.1. The data are represented as means±S.E.M. from groups of six to ten animals. The data illustrate that there is considerable batch to batch variability in control values, and emphasized the necessity to include adequate controls with each experiment.

4.2. Drug levels

Drug levels were assayed with either a gas chromatographic procedure in the cases of IMI and FLU or by radiochemical assay (for MAO activity) in the case of TCP. Data are presented in Table 4.2. These levels (or the degree of inhibition of MAO activity with TCP) are similar to these observed by other researchers in the Neurochemical Research Unit, Department of Psychiatry in the University of Alberta (McManus *et al.*, 1992, Goodnough and Baker, unpublished data, 1994; Todd *et al.*, 1995).

Table 4-1. PKC and PKA activities in rat cortex and hippocampus. Brain regions were dissected from control and drug treated rats as described under Materials and Methods. The data represent the mean enzyme activities (±S.E.M.) as pmol phosphate transferred to histone per mg sample protein for 30 min, measured in cortex and hippocampus samples after 14 days exposure to antidepressant drug.

Table 4-1. PKC and PKA activities in rat cortex and hippocampus

PKC activity in cortex (pmol phosphate/mg protein)

Fraction	Control	TCP	IMI	FLU
Soluble	147.4±12.0	78.2±6.6	72.6±6.5	-
	96.4±5.3	-	-	125.9±8.2
Particulate	14.6±1.0	26.0±2.6	26.8±2.9	-
	6.9±1.7	-	-	5.3±0.6

PKC activity in hippocampus (pmol phosphate/mg protein)

Fraction	Control	TCP	IMI	FLU
Soluble	31.6±1.5	-	-	25.1±1.9
Particulate	7.0±0.5	-	-	9.5±0.5

PKA activity in cortex (pmol phosphate/mg protein)

Fraction	Control	TCP	IMI	FLU
Soluble	37.8±0.3	30.9±2.1	68.4±6.6	-
	19.4±0.5	-	-	13.3±0.9
Particulate	14.7±1.2	15.5±3.0	12.0±0.9	-
	7.5±0.4	-	-	9.5±0.7

PKA activity in hippocampus(pmol phosphate/mg protein)

Fraction	Control	TCP	IMI	FLU
Soluble	8.3±0.9	20.3±1.5	20.4±1.8	-
	11.8±1.8	-	-	18.7±2.4
Particulate	10.6±1.5	9.7±1.2	12.3±1.2	-
	14.5±1.4	-	-	9.8±0.7

Table 4-2 Drug concentrations or MAO activity in brain tissue

TCP	% inhibition of MAO-A 87.7% ±1.5
	% inhibition of MAO-B 93.5% ±2.1
IMI	496.0±70.9 ng/gm tissue
DMI	323.4±37.8 ng/gm tissue
FLU	2.0±0.5 µg/gm tissue
NORFLU	14.7±5.1 µg/gm tissue

Data are the mean ± S.E.M. from 7-12 animals.

5. DISCUSSION AND CONCLUSIONS

5.1. Discussion

5.1.1. Reliability of methods

The methods used in the present work consist of preparation of fractions of rat brain containing the enzymes of interest and estimation of their kinase activities utilizing a paper affinity chromatography-protein phosphorylation assay. The principal aim of the extraction procedure is to avoid loss of the enzymes of interest, consistent with the retention of their catalytic activity. Mechanical disruption of cell integrity after loose homogenization (modified from Nestler and Tallman, 1988; Mann *et al.*, 1995), as used here, has been shown to provide good tissue disruption and separation of cells and subcellular structures and is in routine use in many laboratories, here and throughout the world. Damage to enzymes can result from a number of causes, such as proteolysis, oxidation of thiol groups, denaturation and loss of activity in solution. To avoid this, all the procedures were carried out at 4°C, after dissection of rat brains, and in the homogenization buffers we used several potent protease inhibitors and heavy metal ion chelators to avoid damage to the enzymes of interest. Sucrose was also included as a free radical scavenger as well as to minimize osmotic shock. The incubation buffer used has a pH of 7.5 which is close to the optimal pH of the enzymes. Possible pitfalls could arise if the homogenates were not cooled sufficiently, so that proteases are activated and hydrolyze other proteins in solution. Utmost care was therefore taken to ensure that tissues were cooled as rapidly as possible and maintained at low temperature until the point of incubation.

The paper affinity chromatography protein phosphorylation assay, utilizing cation-exchange paper, was first used by Witt and Roskoski (1975a,b) and later by Nestler and Tallman (1988) and Nestler *et al.* (1989). In comparison with the phosphocellulose-paper method, the assay has been shown to be superior for basic proteins (as in the case of histone). The assay can process a large number of samples, simultaneously, in the same container; the procedure is rapid and simple and the materials are relatively inexpensive. Loss of radiolabelled protein from the paper during the washing procedure was a concern, and a

number of trials were held to establish the optimum concentrations of TCA and ethanol, and durations of the wash periods at each stage. The protocol that was finally adopted and described in Methods provided reproducible results with a variability within 5%. Possible experimental errors that may occur include contamination of paper squares with skin secretions, when touched with bare hands, and contamination of counting vials with radioactive material due to mishandling the isotope, and these may be obviated with commensurate care. The experimental protocol is one that requires experience to achieve the levels of reproducibility that were a feature of the present work. The required expertise was obtained during a series of work-up experiments and the data obtained are at least comparable in accuracy to that published by other laboratories working in the area of protein kinase activity.

In all the experiments, simultaneously with treatment animals, controls were implanted with minipumps delivering 0.9 % saline. The enzyme activities in test preparations were compared at all times to the control levels in order to minimize the possible errors. Finally appropriate statistics were employed to ensure the homogeneity of population responses and the significance of the differences observed.

5.1.2. Comparison of kinase activities with literature data

At rest PKC is mainly located in the cytoplasm (with a prevalence of conventional α and β isoforms over novel ε and δ) and it is catalytically inactive. It becomes fully activated by the concerted action of DAG, Ca^{2+} and acidic phospholipids (PS) and translocates mainly to the cellular membrane. Therefore the finding of increased activity in the particulate or membrane-associated fraction could be regarded as a true activation of the enzyme. In the same way the observed increase of the cytosolic fraction of PKA could also be regarded as an increase in enzymatic activity, keeping in mind that when activated, the catalytic subunits dissociate from the regulatory subunits of the enzyme and are found mainly in the cellular cytoplasm.

The levels of PKC activity observed in the present experiments are comparable to

those observed by several authors prominent in the field (O'Brian *et al.,* 1984, Caravatti *et al*., 1994, Mann *et al.* 1995) and we can be confident, therefore, that our data can be compared with the existing literature for PKC. Similarly, the PKA activities obtained in the present work agree well with the level of activity reported by Hidaka *et al.* (1984), but are lower than those reported by Nestler (Nestler and Tallman, 1988, Nestler *et al.*, 1989). This latter discrepancy is consistent in that Nestler habitually reports higher levels of activity that other authors. The agreement of the results obtained in the present work with the levels of activity reported by the prestigious and prolific Hidaka group encourages us to believe that there is no significant deficit in the methodology employed here.

The trends observed in the current experiments with PKA are in broad agreement with several authors (Perez *et al.*, 1989, Mori *et al.,* 1998, Tadokoro, *et al.,* 1998). There is a consensus that tricyclic antidepressants elevate PKA activity in the soluble fraction of several brain areas. The same could be said for SSRI's, although the level of agreement is less and dependent on the area of the brain studied.

The picture is more clouded with PKC. While our observations suggest a shift of activity from soluble to particulate caused by IMI and TCP and an overall increase in soluble activity in response to FLU in the cortex, and a modest shift from soluble to particulate in response to FLU in the hippocampus, both Dwivedi and Pandey (1997a) and Mann *et al.* (1995) reported reduced PKC activity in both cytosolic and membrane-associated fractions from cortex and hippocampus following chronic daily intraperitoneal administration of desmethylimipramine, phenelzine or fluoxetine. Tadokoro *et al.* (1998) on the other hand found no change in PKC activity of either the particulate or soluble fraction in either cortex or hippocampus of animals treated with IMI or sertraline (an SSRI). Li and Hrdina (1997) reported similar results for FLU or DMI using GAP-43 phosphorylation in the cortex as an index of PKC activity, The reason for these discrepancies is not immediately clear. The possibility exists that the effects are drug specific and are unrelated to the common therapeutic effect of all of these drugs. Some evidence for a direct effect of the residual drug on PKC does exist and this possibility cannot be excluded from consideration. Nalepa and Vetulani (1991) and Morishita and Watanabe (1997) indicated that tricyclic antidepressants

had a direct inhibitory effect on at high concentration. A further possibility for this discrepancies in this area may be the amount of chelator used in homogenization buffers. For instance, Tadokoro *et al.* (1998) used 10 mM EGTA and 5 mM EDTA (for comparison we used 3 mM EGTA and did not use EDTA), which in such large amounts are known to chelate not only the heavy metal ions in the solution, but also the Zn^{2+} ions needed for protecting the activity and native form of PKC. Our data are in agreement with Szmigielski and Gorska (1997) who found that chronic administration of imipramine increased the activity of membrane-associated fractions of PKC in both rat frontal cortex and hippocampus for a 21 day period, using the same dose of imipramine as in the present study.

Chronic treatment with several classes of antidepressants has been reported to enhance the covalent binding of [^{32}P]-cAMP to the regulatory subunits of PKA in the soluble fraction of the rat cerebral cortex (Perez *et al.*, 1989; 1995). Binding of cAMP to the regulatory subunits of PKA promotes the release of the free catalytic subunit and thus promotes the phosphorylation of specific substrate proteins.

5.1.3. Drug levels measured in brain.

A feature of the present work that distinguishes it from the existing literature on the subject of antidepressant effects on enzyme levels is that close attention was paid to the levels of drug measurable in the brain immediately *post mortem*. The favoured route of administration of antidepressant drugs to experimental rats has been by intraperitoneal injection once or twice a day (Nestler *et al.*, 1989, Nalepa and Vetulani, 1991, Mann *et al.*. 1995, Dwivedi and Pandey, 1997b, Mori *et al.*, 1998). Such a protocol, while differing little from the therapeutic regimen for the three drugs in question, does tend to produce marked fluctuations in plasma concentrations over a 24 hr period, at least in the case of imipramine. The use of Alzet® minipump technology ensures delivery of drug over the time scale of the experiment at only a modestly declining rate (Klassen, 1998). In this way we can be sure that the exposure of the tissues remains relatively constant and suitable for studying any adaptive changes that might occur. As noted in the text, the data are in concert with the

results of others working in the same animal system in the same laboratory (McManus *et al.*, 1992, Goodnough and Baker, unpublished data, 1994; Todd *et al.*, 1995) and from others (Evreux *et al.*, 1968, Petty *et al,* 1982, Pohland *et al.*, 1989). While this is reassuring, the question remains whether the levels of drug in the brain are in any way relevant to those experienced clinically. This is a difficult parameter to establish for obvious ethical reasons. Certainly the near but not quite complete inhibition of MAO by TCP indicates that the level of drug was adequate to achieve a diagnostic end point, without venturing into the serious overdose range. Insofar as IMI or FLU are concerned, the picture is less clear with respect to human comparators. However, there are some indications from *post mortem* suicide data. Both IMI and FLU concentrate in the lung both after a single dose in the therapeutic range and during suicide attempts with overdose (Pohland *et al.*, 1989, Jones and Pounder, 1987). The levels seen in brain are regarded as intermediate among tissues levels. The recorded combined level of IMI and its metabolite, desmethylimipramine, were around 50μg/g in brain tissue from a suicide victim. This is over 50 fold greater a level than measured in the present work but would be consistent with a fatal overdose. It would appear therefore that the tissue levels of IMI at least measured in the rat brain in the present work are considerably below the level at which toxicity (usually cardiac) occurs, and represent a reasonable approximation of levels attained during clinical use.

5.1.4. PKC vs PKA

Protein phosphorylation-dephosphorylation is recognized as a major regulatory mechanism of extracellular signaling and transduction. There is extensive evidence of the importance of protein kinases, phosphatases and phosphorylated proteins in regulation of neuronal functions, and in particular the interaction between various protein kinases in the regulation of cell function..

From earlier studies it appears that PKC is mainly associated with the particulate fraction in brain while PKA is generally a soluble enzyme (Walaas *et al.*, 1988; Walaas and Greengard, 1991). The results in this thesis show an increased PKC activity in the

membrane fraction from the cortex following treatment with TCP and IMI which is commonly taken as an indication of increased enzyme activation. There was not a consistent alteration of the activity of membrane fraction of PKA in the cortex at the same time and the conclusion can be drawn that there was no evidence for significant interaction between the enzymes at the gross macroscopic level.

In the hippocampus, there was a modest shift of PKC from the soluble to the particulate fractions, possibly indicating enzyme activation, following treatment with FLU. Concurrently, the activity of PKA in the soluble fraction from the hippocampus was also increased with all three drugs studied. This does not constitute evidence of dependence or interaction, but it is an interesting coincidence and suggests that levels of protein phosphorylation in the hippocampus of antidepressant treated animals are actually or potentially increased.

5.1.5. Disease states

A question that arises from the present work is to what extent these effects are representative of the therapeutic benefit conferred by the drugs, and as a corollary, do these data shed any light on the etiology of depression itself. An increase in PKC activity in the cytosolic fraction of platelets from patients diagnosed with major depression was found by Pandey *et al.* (1998), suggesting an increased PKC synthesis or decreased translocation since no changes in the activity of the membrane fraction was observed. However, a decrease in the activity of PKC of both membrane and cytosolic fractions of brains from suicide victims was observed by the same authors (Pandey *et al.*,1997). The present work indicates that drugs with the same therapeutic endpoint have disparate effects on the levels of activation of PKC. Indeed, the SSRI had an effect that was opposed to that produced by the other two drugs. As their name implies, SSRI's are selectively effective on serotonin uptake, whereas the effects of the TCAs and MAOIs are more broadly applied to monoamine uptake in general and noradrenaline uptake in particular.

Abnormalities were also observed in the activity of PKA. Patients with untreated

depression were investigated and showed significantly lower activity of the cytosolic fraction of PKA in skin fibroblasts than do normal subjects (Shelton *et al.*, 1996); these workers suggested that these effects could be a result of differences in either the regulatory or the catalytic subunit of PKA. It should be noted that there is general agreement in the literature, and from the present work, that PKA activity in the soluble fraction, i.e. the functional enzyme in the cell, is generally elevated by antidepressant drugs (see section 5.1.2). It is a long way from this observation of a coincidence to asserting that the etiology of depression involves, or even stems from, a deficiency in PKA activity. Yet the suggestion has been made (Racagni *et al.*, 1992) and the present data merely serve to reinforce the concept. It is entirely possible that the disparate results of PKC activities reflect a relative dissociation between this enzyme and its activity and the disease of depression. The same cannot be said for PKA.

5.1.6. Possible implications

The observed shift of the enzyme activities in our results might be on one hand a consequence of antidepressant application in therapeutic doses and being important in the clinical effects observed on antidepressant administration. The shift of the total activity of the enzymes could be on account of increased expression and synthesis or decreased degradation of PKA in the hippocampus with all the drugs studied, and a corresponding decrease in the total activity of PKC in the cortex following TCP and IMI. Specific studies on gene expression are required to elucidate this point.

PKC activation and phosphorylation of its substrate proteins seem to promote neuronal growth and development indirectly, increase the production and release of endogenous neurotransmitters, enhance axonal and dendritic elongation and remodel gap junctions. All that may counteract stress-induced atrophy of vulnerable neuronal populations, which is observed in susceptible individuals.

The observed increase of soluble PKC activity with SSRIs might be attributable to an increase in enzyme synthesis, or a failure of the soluble enzyme to translocate to the

membrane either through a deficit in the translocation signal, or an inherent unresponsiveness in the system. Since this is not an uniform response, the likelihood that this system has little bearing on the etiology of depression or its therapy is considerable.

The observed increase of soluble cortical PKA activity only with IMI is interesting, but may reinforce the impression that the main seat of affective illness lies at subcortical levels, and that results from the cortex are a poor predictor of therapeutic effect.

The results from this study are in agreement with the hypothesis of Duman *et al.* (1997) and Wachtel (1989). The first researcher stated that long-term antidepressant treatment result in the sustained activation of PKA in certain populations of neurons in the hippocampus and cerebral cortex. That alteration of PKA activity leads to regulation of specific target genes in the above areas, including the increased expression of brain-derived neurotrophic factor and tyrosine kinases pathway. That neurotropins prevent the atrophy of stress-vulnerable hippocampal neurons and promote their survival and growth. Therefore the most important antidepressant effect might be increased activity of the PKA cascade in hippocampus, which has implication on expression on certain target genes and respective proteins.

Wachtel in his turn hypothesized the core abnormality in depression to be the imbalance of PKA and PKC cascades, resulting in an underfunction of the PKA pathway, associated with a dominance of the PKC pathway. This hypothesis is supported by the ability of all antidepressants to enhance monoamine availability in the synaptic cleft through different mechanisms.

The observed changes in PKC activity in this work, while not entirely consistent with each other, may merely serve to separate the PKA and PKC cascades from each other in terms of Wachtel's ideas, and place relatively greater emphasis on PKA, in line with Duman's suggestion.

5.2. Conclusion

Although our understanding of antidepressant action beyond the receptor level has improved recently, the investigation of alterations in brain protein kinase activities in conjunction with different antidepressant drugs is still a challenging area for investigation. Two kinases, PKC and PKA, have been identified as major intracellular targets for the long-term effects of antidepressant drugs.

A significant finding of this work is that there is no obvious overall pattern of protein kinase alteration common to two areas of brain which are thought to contribute to the clinical efficacy of antidepressant drugs. However the increase in activity of PKA in the soluble fraction of the hippocampus produced by all three antidepressants is of interest and should be pursued in future studies on other antidepressants to determine if it may represent an useful measure of screening for probable antidepressants in future.

A corollary to changing levels of protein kinase activity is that the degree of substrate phosphorylation is affected. Dynamic changes in the phosphorylation of functional cellular proteins by brain protein kinases may well be involved in the therapeutic mechanism of antidepressants. The importance of the increased activity of both pathways either in concert or separately may lie in the direction that it points future research into the etiology and treatment of the disease.

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